THE DUAL ROLE OF INTERLEUKIN-6 IN EXPERIMENTAL AUTOIMMUNE MYOCARDITIS AND DILATED CARDIOMYOPATHY

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A dissertation submitted to Johns Hopkins University

in conformity with the requirements for the degree of

Doctor of Philosophy

Baltimore, Maryland

April 2015

Abstract

Myocarditis is a leading cause of sudden death in young adults. With its associated dilated cardiomyopathy (DCM), myocarditis accounts for 45% of all heart transplants in the United States. The mechanisms driving the progression of myocarditis and DCM to heart failure are largely unknown. The goal of my research has been to elucidate this mechanism. Increased interleukin-6 (IL-6) levels are associated with heart failure and are found in patients with DCM. The results presented in this dissertation define the mechanisms by which IL-6 induces myocarditis and drives dilated cardiomyopathy employing the mouse model of experimental autoimmune myocarditis (EAM). Using antibodies to the receptor of IL-6 (IL6R) and mice genetically deficient in IL-6 (IL-6KO), we show that IL-6 is required to induce EAM. Furthermore, in experiments where IL-6KO mice were treated with timed doses of recombinant IL-6, we establish that there is a requirement for IL-6 in during the initial priming phase of EAM. Using different adjuvant preparations, we establish that the main distinction between a benign immune response and a pathogenic immune response to the heart is partially due to the production of IL-6. By interrogating chemokines and cytokines produced, and the cellular response in the draining lymph node, we further demonstrate IL-6 induces dendritic cell (DC) trafficking to the lymph node by DC CCR7 expression and lymph node CCL21 expression. We then sought to describe the mechanism by which IL6 drives the progression of established EAM to DCM. The use of anti-IL-6R antibodies established that IL-6 is

required for the development of DCM. Hydrodynamic delivery of a plasmid encoding IL-6 to mice with EAM concluded that IL-6 drives changes in the functional parameters associated with heart failure while sparing further cardiac fibrosis. Utilizing myocyte-restricted gp130$^{-/-}$ mice, GP CKO, we found that the requirement for IL-6 in DCM is through signaling on the myocyte. We further illustrate that IL-6 signaling to the myocyte during DCM induces changes in the expression of proteins related to myocyte function, demonstrating the pathogenesis of IL-6 in DCM is attributed to signaling on the myocyte leading to loss of myocyte function. Together, these results establish for the first time a dual role for IL-6 in EAM and DCM - first in the induction of an appropriate innate response during the priming phase of EAM and second as a direct driver of decrements in cardiac myocyte function. These findings suggest that local manipulation of IL-6 levels could present therapeutic targets. Early targeting of IL-6 responses could be used to fine-tune an immune response to immunization in the context of vaccination. Heart-specific targeting of IL-6 signaling during heart failure could potentially protect myocyte function.

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Preface

Science builds on the knowledge that precedes it, and I believe scientists develop in the same manner. I owe a great debt to the many friends and advisers that I have blessed to know during my PhD years.

It is with my deepest gratitude that I would like to thank my mentors, Dr. Noel R Rose and Dr. Daniela Čiháková for the opportunity to study under their guidance during my doctoral research. They provided the scientific environment and intellectual stimulation for my development as an immunologist. Without their knowledge and encouragement, this work could not have been possible.

I would also like to thank my fellow lab mates, whose assistance and advice has been essential to my success over these past years. Upon entering the lab, Drs. Christian Baldeviano and Jobert Barin introduced me to the research methods of the laboratory, which was critical to my understanding and use of our animal model. Two other students, Drs. SuFey Ong and Lei Wu, entered the doctoral program, and laboratory, around the same time as I did. They have both been immensely helpful over the years for all the day-to-day necessities. Through everything from figuring out protocols to learning flow cytometry, SuFey and Lei were always around to help, or at least to be just as lost alongside me. I would like to particularly thank SuFey for years of conversation both scientific and otherwise, she has been the best office-neighbor during my research. Thank you as well to Nicola Diny, Hee Sun

Choi, Snow Hou, Julie Schaub who have all been helpful in my experiments and sometimes more importantly, in making our lab a great environment in which to work.

I would also like to thank other members of the lab. Monica Talor has assisted with experiments, ran assays, and in general has kept the lab running so that the rest of us could do our jobs well. Monica has provided immeasurable help over the years for which I am very thankful. I would like to thank past members of the lab, with whom I spent time working alongside over the years. Thank you to; Dr. Quan Nhu, Natalie Stickel, Ashley Cardamone, Elizabeth Gebremariam, Rob Horel, Drs.Ping Chen, and Davinna Ligons.

My work was also made possible with help from many collaborators and core facililtes. I would like to acknowledge Drs. David Kass and Djahida Bedja for their assistance with mouse echocardiography; Xiaoling Zhang and Lee Blosser for their support in flow cytometry; Dr. Kirk Knowlton and Sasha Brooks for providing transgenic mice. I would also like to acknowledge my floor-mates in the Ross Research Building, Drs. Melissa Salgado, Alessandra De Remigis and AeRyon Kim, for their help with reagents, instruments and coffee over the years.

I would like to thank the professors who have been great sources of guidance and feedback for the course of my research. Thank you to my thesis advisory

committee, Drs. Noel R Rose, Daniela Čiháková, Alan Scott and Fengyi Wan. I would like to thank Dr. Alan Scott for years of support, from serving on exam committees to directing my study of immunology, to general conversations about managing the experience of pursuing a PhD. My experiences at Johns Hopkins have been greatly enhanced through my interactions with Dr. Scott. I would also like to thank another member of my thesis committee, Dr. Fengyi Wan, who has provided excellent scientific guidance at critical points of my research.

I would like to thank mentors that have developed my passion for teaching throughout my PhD. Thank you to my mentor, Dr. Noel R Rose for encouraging my passion for teaching, and allowing me to pursue that passion while I was working in his lab. Thank you to Dr. Christian Coles for allowing me to act as a teaching assistant for years in his course, and for being a source of guidance in developing my own course as I transitioned into an instructor myself. Thank you to the Gordis Teaching Fellowship program of the Johns Hopkins University Public Health Studies undergraduate program for allowing me the opportunity to teach my own course. Teaching throughout my PhD has been a constant source of happiness for me, and I will forever be grateful for all the support and experiences.

Pursuing a PhD involves many experiences outside of labs and classes. I would like to thank other students with whom I have studied, learned, and laughed. Thank you to; Stefanie Trop, my favorite PhD student / fitness

instructor; to Andrea Hodgson for conversation, advice and most recently - help with Western blotting, to Dr. Benjamin Blumberg for being a great friend both inside and outside of school. Thank you especially to Dr. Kely Sheldon and Peter Dumoulin for being incredible friends. Thank you to Samantha Baer for being my study partner, teaching partner and source of sanity.

Thank you to my family, and in particularly, to my father Ken Legault. My dad has encouraged my passion for education through years of study, and has fostered my love of science through his endless support and friendship.

Finally, thank you most of all to my best friend and husband Trevor Fontes. Trevor has supported me through every moment of my PhD, from applying to Johns Hopkins to putting together this dissertation. His love and friendship has strengthened me throughout this journey.

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List of abbreviations

DCM	Dilated cardiomyopathy
EAM	Experimental autoimmune myocarditis
IL	Interleukin
IL-6KO	IL-6 knock-out mouse
DC	Dendritic cell
GP CKO	Gp-130 cardiac-restricted knockout mouse
CT-1	Cardiotrophin-1
CLC	Cardiotrophin-like cytokine
CNTF	Cilliary neurotrophic factor
LIF	Leukemia inhibitory factor
NPN	Neuropoietin
IL-6R	IL-6 receptor
sIL-6R	Soluble IL-6 receptor
SOCS	Supressor of cytokine signaling
CHD	Coronary heart disease
CVD	Cardiovascular disease
MI	Myocardial infarction
RA	Rheumatoid arthritis
EAE	Experimental autoimmune encephalitis
HF	Heart failure
LV	Left ventricle
CFA	Complete Freund's adjuvant
IFA	Incomplete Freund's adjuvant
MyHC	Myosin heavy chain
EF	Ejection fraction

SERCA	Sacroendoplasmic reticulum calcium transport ATPase
ANP	Atrial natriuretic peptide
BNP	Brain natriuretic peptide

Chapter 1: Introduction

1.1 Myocarditis: Basic Biology

Myocarditis is inflammation of the myocardium [1]. The etiology of myocarditis varies from viral to bacterial to fungal to parasitic to chemical. Viral infection with Parvovirus 19 or Coxsackie B virus accounts for most of the infectious causes of myocarditis [2, 3]. Bacterial infection of *Borrelia burgdorferi* has resulted in myocarditis and, additionally, parasitic infection of *Trypanosoma cruzi* has been reported to cause myocarditis as well as heart dilation [2] Cardio-toxic chemicals such as 5-fluorouracil, as well as drugs such as doxorubicin have also been associated with myocarditis [4, 5].

Regardless of etiology, myocarditis is the result of immune cell infiltration and damage to the myocardium. Autoimmune myocarditis is hypothesized to develop due to the damage to the myocardium exposing auto-antigens combined with the already activated immune system due to infection or other injury to the heart [2, 6]. Some infections agents, such as *Trypanosoma cruzi*, also express foreign antigens very similar to cardiac epitopes suggesting that molecular mimicry may be one mechanism initiating the autoimmune response to the heart [7].

The evidence for autoimmune induction in myocarditis is strong both in patients and animal models. In patients, autoantibodies directed against the heart have been identified, CD4+ T cells have been isolated and have been shown to be active against heart auto-antigens and additionally, immunosuppressive therapy has been beneficial in patients with strongly

suspected autoimmune myocarditis. Mouse models of myocarditis have been developed using viral induction with Coxsackie B viral infection, parasitic induction with *Trypanosoma cruzi* as well as autoimmune induction with myosin immunization [8-10]. The strongest animal-model evidence for the autoimmune induction of myocarditis comes from T cell transfer experiments, as myosin-reactive CD4+ T cells are sufficient to induce myocarditis [11-13].

1.2 Myocarditis: Clinical

Many patients with myocarditis present with general flu-like symptoms, which make myocarditis a difficult disease to diagnose and generally one of exclusion [1, 14, 15]. Other patients may not display any overt symptoms but succumb to sudden death and are diagnosed post-mortem [1]. Myocarditis can be profiled using blood tests for cardiac troponin I and creatine kinase MB, by echocardiography, and by cardiac MRI [2]. Endomyocardial biopsies are required for the gold standard of diagnosis, the Dallas Criteria, which defines myocarditis as the presence of mononuclear infiltration and cardiomyocyte necrosis upon histopathological examination of heart tissue [16, 17].

Most patients that present with myocarditis will resolve their inflammation and will fully recover [18]. The subset of patients that do not, often develop a suspected autoimmune reaction to their heart leading to chronic, autoimmune, myocarditis [19, 20]. Autoimmune myocarditis, can be subtyped by biopsy, which reveals the types of immune infiltration into the heart

(Table 1.1). Giant-cell myocarditis is strongly indicative of an autoimmune reaction and in general is highly associated with progression to heart failure [8, 21, 22]. Patients with eosinophilic myocarditis are at risk of rapid progression to heart failure or sudden death [23]. The most common sub-type is lymphocytic myocarditis, and still other patients will have a mixed-cellular infiltration. Approximately a fifth of patients with myocarditis will develop dilated cardiomyopathy [24]. The mechanisms driving this progression to heart failure are largely unknown and are the topic of the third chapter of this dissertation.

1.3 Inflammatory dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is characterized by an enlarged heart that ultimately results in an ineffective pumping capacity and leads to heart failure [25]. DCM that is associated with a previous inflammatory response in the heart, as in myocarditis, is additionally characterized by fibrotic tissue [14]. Fibrosis of the heart results from remodeling of the tissue following the dissipation of immune cell infiltration. DCM is the leading cause of non-congenital cardiomyopathy in patients under the age of 40 [26].

Treatment for patients with DCM is largely based on symptom management. General heart failure medications such as beta-blocks and ACE inhibitors are used to manage heart function [27]. There is no cure for DCM. The only permanent treatment of DCM is heart transplantation and myocarditis and the associated DCM is the reason for approximately 45% of all heart

transplantation in the US [28]. For patients with a known history of myocarditis and suspected autoimmune associated DCM, immunosuppressive drugs azathioprine and prednisone have been used experimentally, with some success [29].

The mechanisms driving the progression from myocarditis to DCM are largely unknown. The third chapter of this dissertation will show how IL-6 drives loss of heart function and present a potential therapeutic target, trans-IL-6 signaling, for mediating this progression.

1.4 Mouse models of experimental autoimmune myocarditis

In order to study the biological mechanisms of autoimmune-associated myocarditis, we utilize a mouse model of experimental autoimmune myocarditis (EAM) (Figure 1.1). EAM is an extensively characterized model that provides a unique tool to dissect the immune and tissue mechanisms of myocarditis. EAM is induced by immunization with a peptide derived from the cardiac myosin heavy chain alpha (MyHCα614-629) in susceptible mouse strains (Figure 1.1). BALB/c, A/J, A.SW, C3H and FVB/NF mice are all susceptible strains that respond to immunization. The BALB/c model has been most extensively characterized and will be the model of this dissertation. Following immunization, mice develop robust immune cell infiltration into the heart staring by day 10 and peaking after 21 days. Subsequently, infiltration will wane and remodeling will begin. By day 35

decreases in heart function can be measured and by day 45 DCM develops (Figure 1.2) [30]. This model provides an invaluable tool to study the progression of disease that mirrors the human course of disease.

1.5 Immune cells of experimental autoimmune myocarditis

EAM is characterized by inflammation of the myocardium with infiltration of immune cells into the tissue. EAM is a T-cell dependent disease [12, 13]. Mice develop auto-reactive antibodies, however, B cells and antibodies are not sufficient to induce disease and do not correspond to disease severity [31, 32]. EAM is driven by auto-reactive CD4+ T cells [12, 13]. Depletion of CD4+ T cells protects mice from EAM and transfer of auto-reactive CD4+ T cells into naïve mice induces EAM [11]. EAM will develop in the absence of any one subtype of CD4+ T cell, as evidenced by the use of knock-out mice which have a deletion in a particular lineage such as IL17AKO mice [33-36]. DCM, but not EAM, requires the Th17 lineage [36].

1.6 Interleukin-6: Basic Biology

IL-6 is a pleiotropic cytokine which bridges the innate and adaptive immune systems [37]. Perturbations or dysfunction in the transition from innate to adaptive immunity have long term consequences for inflammation and autoimmunity [38]. The acute response to IL-6, which is largely protective, to chronic, long term signaling leading to pathogenic inflammation and autoimmunity is an example of the varying faces of IL-6 [39].

IL-6 has a wide array of biological functions and is produced by many cells of the body. Originally identified as a B-cell differentiation factor, IL-6 is now recognized as a cytokine that regulates many processes such as the acute-phase response, inflammation and hematopoiesis. It can be made by most tissues as well as virtually all cells of the immune system. IL-6 can signal either through membrane-bound receptors or, uniquely within the IL-6 family of cytokines, can signal in trans, with a soluble form of its receptor (Figure 1). IL-6 has been shown to participate in neurogenesis, wound healing and hepatic regeneration [40-42]. Acutely, IL-6 responds to almost all perturbations of homeostasis. However, when IL-6 remains elevated chronically, the protective roles of IL-6 in maintaining tissue integrity and signaling in the immune response are no longer required and constant signaling becomes associated with fibrosis and chronic inflammation. This dual role of IL-6, from acute and beneficial to chronic and harmful, is the subject of this dissertation.

IL-6 specific biologic functions and signaling

IL-6 is a member of the IL-6 family of cytokines that also includes IL-11, IL-31, cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), cilliary neurotrophic factor (CNTF) leukemia inhibitory factor (LIF), neuropoietin (NPN), and oncostatin M [43-45].

As a multi-functional cytokine, IL-6 acts on the immune system as well as other local tissues. Within the immune system, IL-6 can direct the

development and activation status of both innate and adaptive immune cells. IL-6 signaling up-regulates anti-apoptotic molecules in T cells [46-48]. In addition, IL-6 is required for Th17-lineage differentiation through STAT3 dependent mechanisms [49, 50]. This is particularly important because the Th17 lineage has been implicated as a contributor to pathogenesis in many autoimmune diseases. IL-6 also has functions in the innate immune system, where it induces the differentiation of monocytes to macrophages rather than dendritic cells [51]. IL-6 may also influence DC activity as it can alter DC CCR7 expression and IL-6 secretion by DCs can affect the immunosuppressive activity of Tregs [52-54], thus bridging the innate and adaptive immune responses. And importantly for the initiation of many inflammatory responses, in the tissue IL-6 suppresses neutrophil infiltration while promoting directed trafficking and activation of mononuclear leukocytes [55-59]. Together, these studies show how IL-6, if not properly controlled, can direct a proinflammatory immune response that can trigger an auto-aggressive response through the Th17 lineage.

The IL-6 cytokine family signals through a cytokine-specific receptor complexed with at least one subunit of the signal-transducing protein, gp130 [44]. IL-6 specifically signals through a complex of the IL-6R (also known as IL-6R-alpha) [60] and the IL-6-family common receptor gp130 [61, 62]. GP130 signaling mediates a variety of cellular processes including cell survival, apoptosis, growth, proliferation, differentiation and survival [63-66].

GP130 is part of the receptor complex for CNTF in the brain, LIF, oncostatin M, NPN, cardiotrophin (CT-1), IL11, IL27 and IL31 [67-73]. Importantly, gp130 is expressed on nearly all cells in the body. Therefore, what gives IL-6 family cytokines tissue-specificity is the cellular expression of the co-receptor for each family member cytokine.

The IL-6R is mainly expressed on hepatocytes and immune cells. However, IL-6 is unique in the IL-6-family because it has a soluble form of its receptor. Therefore, cells lacking the IL-6R can still respond to IL-6 because the naturally occurring soluble form of the IL-6R exists and can create a complex with IL-6 (Figure 1.3). IL-6 first binds to the IL-6R and this complex of IL-6 and IL-6R then binds with gp130 [74, 75]. The soluble IL-6R (sIL-6R) is generated either by cleavage of the membrane-associated receptor or, independently, by translation of an alternatively spliced mRNA [76-78]. This signaling of the sIL-6R and the membrane bound gp130 is referred to as IL-6 trans-signaling [79] (Figure 1.3). Trans-signaling has been shown to be active in many systems where cells only become responsive to IL-6 in the presence of the sIL-6R, such as in hematopoietic progenitor cells [80, 81], T cells [82, 83], and endothelial cells [84].

Downstream signaling of the IL-6R combined with gp130, whether soluble or membrane bound, signals through JAK-STAT, Ras-MAPK, or PI3K, pathways [85, 86]. Within the JAK-STAT pathway, IL-6 specifically signals through STAT3, which dimerizes and then translocates to the nucleus [87].

Regulation and termination of downstream IL-6 signaling is mediated through suppressor of cytokine signaling (SOCS) proteins [88-90]. The negative regulator of IL-6-STAT3 activation, SOCS3, may in part regulate the protective versus pathogenic effects of IL-6.

1.7 The role of interleukin 6 in cardiac inflammation and cardiac failure in humans

Population based studies

Meta-analysis of human studies has demonstrated that long-term elevation of IL-6 levels more than double a person's life-time risk of coronary heart disease [91]. These studies, among many others, demonstrate an association between pathology and chronic IL-6 levels. Recent studies have established a causal role of increased IL-6R protein levels in coronary heart disease (CHD) [92-94]. Not understood, is whether elevated IL-6 was a byproduct of the cardiovascular disease (CVD) or was serving a pathogenic function. Whereas association studies have suggested that long-term elevation of IL-6 levels have adverse consequences for cardiac health, these 2 studies have finally given clear evidence that IL-6, a proinflammatory cytokine, plays a causal role in determining CVD risk (Table 1.2). The studies focused on a genetic variant in the population that is associated with increased IL-6 levels circulating in the blood, but decreased IL-6R signaling. Interestingly, these groups went on to look at the effect of the variant compared to the anti-IL-6R drug, tocilizumab, and found that the variant was associated with the same biological changes as the inhibiting drug. The findings of these studies

suggest that targeting IL-6 or, in particular IL-6R-mediated signaling, may be a possible therapeutic intervention for CVD, including a possible preventative measure in high risk individuals.

On a population level, the many polymorphisms in the IL-6 promoter region as well as polymorphisms in the IL-6R gene locus are associated with inflammation and increased disease risk [95-98]. A particular polymorphism in the promoter region of IL-6 was shown to lead to higher systemic levels of IL-6 [95-97]. This variant is uniquely associated with susceptibility to systemic juvenile idiopathic arthritis and importantly, led to the use of anti-IL-6R antibody for its treatment [98-101]. The polymorphisms associated with elevated protein levels of IL-6R are also associated with inflammation and are predictive of adverse coronary outcomes such as cardiovascular disease [93] and abdominal aortic aneurism [102]. Elevated IL-6 serum levels in patients may be predictive of poor outcomes, thus providing a potential prognostic tool, in a variety of heart-related diseases such as heart failure, myocardial infarction (MI), and angina [103-106]. Human studies clearly implicate IL-6 signaling in the heart to be pathogenic over time, however some experimental data using animal models of acute insult to the heart, contradict these associations. The final outcome of IL-6 signaling seems to be greatly dependent the duration of the signaling, as well as the downstream signaling cascades activated.

The identification and description of IL-6 trans-signaling has begun to explain how IL-6, uniquely in the IL-6-family, has been shown to be protective in acute inflammation and disease such as septic shock but pathogenic in chronic disease [107-111]. Early in the study of IL-6, chronic overproduction of the cytokine was implicated in the pathogenesis of many inflammatory conditions including rheumatoid arthritis (RA), Castleman's disease and cardiac myxoma [112-114]. In all these disease states, a constitutively increased IL-6 level explained the pathogenic inflammatory symptoms of the patients. Because of this, a therapeutic antibody targeting IL-6 signaling, anti-IL-6R, which targets membrane-bound as well as a soluble receptor, has been used to treat RA in small studies of Castleman's disease and multiple myeloma [115-117]. Targeting the IL-6R has been shown to be particularly effective in clinical trials for severe RA [118-120] and Crohn's disease [121] which is important because IL-6 trans-signaling is particularly implicated in these diseases where high levels of sIL-6R have been found in patients [122-125] and associate with worse disease outcomes [125]. Trans-signaling of IL-6 may be more common in chronic IL-6 pathology and thus a way to target chronic signaling in the long-term, while preserving classical IL-6 signaling, which is required during acute tissue insult.

IL-6 in mouse models of inflammatory disease

IL-6 is pathogenic in a variety of inflammatory conditions in mouse models (Table 1.3). IL-6KO mice are resistant to experimentally induced RA [126], colitis [82], experimental autoimmune encephalitis (EAE) [127], experimental autoimmune myocarditis (EAM) [128] and autoimmune kidney disease [129]. Additionally, antibodies that target IL-6 signaling block the development of many of these same diseases. IL-6R blockade ameliorates colitis [82], inhibits the onset of autoimmune kidney disease [130] and delays the development of collagen induced arthritis [131]. How exactly IL-6 is exerting its effects in each model may show a role for signaling both to the immune system as well as the local affected tissue.

Evidence for the role of local, tissue-specific IL-6 signaling in the pathogenesis of chronic inflammatory diseases comes from mouse studies that specifically target IL-6 trans-signaling. The argument can be made that whole-animal knockouts of IL-6 or systemic blockade of IL-6 have many effects and thus the specific role that IL-6 is contributing cannot be teased away from these off-target effects. However, by targeting trans-signaling, classical IL-6 signaling is preserved, therefore only cells that do not express the IL-6R are impacted. In many studies this translates into the local blockage of IL-6 signaling in the tissue by blocking IL-6 trans-signaling as immune cells have the IL-6R. In one study of renal pathology in lupus-prone mice, an inhibitor of trans-signaling, sgp130Fc was overexpressed in Lyn-deficient mice and its effect on lupus-associated pathology was measured. IL-

6-deletion in Lyn-deficient mice leads to decreased inflammation, decreased autoantibodies and decreased nephritis [132]. In the sgp130Fc mice, that have classical IL-6 signaling but lack trans-signaling, there was no changes in immune cells, however, there was significantly attenuated glomerulonephritis and improved renal function and reduced complement fixation, showing a role for IL-6 in the local kidney response [132]. Additional studies support these findings in other mouse models. It has long been known that IL-6KO mice do not develop RA [133] but further studies have shown that targeting IL-6 trans-signaling ameliorates RA [134]. Methods to target the local response to IL-6 have been developed, such as tissue-restricted IL-6 production where the whole animal is an IL-6KO except for a tissue of interest. CNS-specific production of IL-6 in a mouse model of EAE showed that IL-6 production in the brain increases inflammatory cell infiltration impairs the blood-brain barrier and worsens disease outcome [135]. Collectively, these studies demonstrate a powerful, pathogenic role of IL-6 in the local tissue that potentially can be therapeutically targeted through the sIL-6R.

IL-6 in the Heart

The cellular response to IL-6 in the heart has been well characterized. Cardiac tissue provides a revealing example where the duration of signaling, from acute to chronic, demonstrates the protective and pathogenic transition (Figure 1.4).

IL-6 family signaling to cardiac myocytes is cardio-protective during the acute response; however, when IL-6 remains elevated chronically, it induces maladaptive hypertrophy and decreases contractile function [136, 137]. Myocytes themselves make IL-6 in response to injury and in addition to increased IL-6 signaling, increased IL-6 production is associated with depressed cardiac function [138]. Acutely, IL-6-family cytokines protect myocytes against oxidative stress and its signaling induces an anti-apoptotic program [137, 139, 140]. However, IL-6-family signaling also depresses the basal contractility of the myocytes as well as the beta-adrenergic responsiveness of the cells leading to decreased function [141]. Prolonged IL-6 family signaling also induces gene expression in the myocytes that is associated with pathological hypertrophy [136]. In chronically IL-6 exposed myocytes, the depressive effects on contractility of IL-6 are mediated by enhancing de novo synthesis and activation of calcium-independent iNOS proteins [142]. Interestingly, the IL-6-driven decrease in contractility was associated with JAK/STAT signaling but not the alternative downstream signaling, ERK pathway, suggesting that differential regulation of downstream signaling is a factor in fine-tuning the cellular response to IL-6-family signaling [142].

The most well characterized protective functions of IL-6 family signaling have been studied in ischemia-reperfusion injury and myocardial infarction which both induce IL-6 production by cardiac myocytes [143-147]. Increased

IL-6 plays a role in late phase pre-conditioning that confers cardio protection [148, 149]. STAT3, the downstream signaling molecule of IL-6, is also required for pre-conditioning [148]. However, chronic elevated myocardial production of IL-6-family cytokines, which occurs post-MI and in HF, have been associated with worse heart outcomes [137, 139, 150]. IL-6 is consistently upregulated in the infarct zone after experimental MI and is associated with left ventricle (LV) enlargement [145, 151, 152]. It is thought that the combined acute effects of IL-6, anti-apoptosis, depressed contractility and hypertrophy, will lead to preserved myocardium in the infarct border zone [145]. Thus, the deleterious effects of IL-6 chronically serve a protective function in MI. Acutely, the combined effects of IL-6 production and signaling by the myocytes leads to preserved cardiac tissue, where damage is limited by reducing the contractility of the cells and inducing an anti-apoptotic program. In the short term these changes lead to smaller infarct zones and thus acute IL-6 is protective in MI. However, both mouse and human studies showed that when this elevated IL-6 continues past the initial requirement to preserve the insulted tissue, these same effects become deleterious. By inducing an anti-apoptotic program and reducing contractility in the long-term, the tissue is less effective as a muscle and begins to induce a genetic program related to hypertrophy, which can ultimately result in heart failure. Thus continued IL-6 signaling is pathogenic.

IL-6 signaling has also been studied from the perspective of limiting or terminating signaling. In cultured cardiac myocytes, overexpression of SOCS3 (limiting IL-6-family signaling) completely suppresses the ability of the IL-6-family cytokines to be anti-apoptotic and induce hypertrophy [153]. This is mirrored in human data where a decrease in myocardium SOCS3 protein expression, which would lead to continuous IL-6 signaling, has also been found in the LV of patients with DCM [154]. Fine-tuning the signal cascade of IL-6 may solve the apparent discrepancy of high IL-6 levels associating with poor heart outcomes, but experimental evidence shows gp130 signaling to be cardio protective. In a mouse model of MI, high levels of IL-6 increased adverse LV remodeling and heart failure because of impaired regulation of the downstream signaling of IL-6, leading to pathogenic, sustained gp-130-mediated STAT3 activation [155]. This study was particularly instructive because the authors identified that signaling through the tyrosine-757 residue on the gp130 receptor mediated these outcomes and lead to prolonged and enhanced JAK/STAT activation, without ERK and Akt signaling, thus pinpointing a specific cascade [155]. The identification of a specific downstream signaling cascade is an important goal because it may identify how or why acute IL-6 is not properly regulated and instead shifts to a chronic signal.

In the myocardium, both chronically elevated IL-6 and increased IL-6R expression lead to continuous activation of gp130, which results in

hypertrophy [140, 156]. To identify the role that STAT3 signaling plays in pathogenic IL-6 signaling, mice were created that over-express STAT3 in the heart. Mice with a cardiac-specific increase in expression of STAT3, the downstream signaling target of the IL-6 cytokines, develop hypertrophy without stimuli [157]. This demonstrates that uncontrolled, continuous STAT3 signaling causes pathogenic changes in the myocardium, independent of initial tissue insult. Alternatively, complete loss of the myocyte expression of gp130 (through cardiac-specific knockout) results in a heart with normal structure and function, although the heart is susceptible to cardiac myocyte apoptosis and dilation in response to pressure-overload [158]. This illustrates the need for IL-6 in the acute response to injury in order to prevent the progression of compensatory LV hypertrophy to heart failure [159].

IL-6 in Myocarditis

Myocarditis provides a striking example of how the dysregulation of normally protective IL-6 responses leads to a pathogenic outcome. During the acute stage of a virus onvection, IL-6 is protective in the heart as it limits viral replication and thus cardiac damage [160]. However, once the virus has been cleared a subset of patients will eventually develop an autoimmune response to their heart and present with autoimmune myocarditis, independent of viral presence [8]. Many of these cases are associated with continuous IL-6 signaling. Over time, patients with autoimmune myocarditis may develop dilated cardiomyopathy, which at its end-stage can only be treated with a

heart transplant [8]. Circulating serum levels of IL-6 increase with the severity of heart failure [161, 162] and upon autopsy, IL-6 has been found to be increased in the heart tissue of patients with DCM [163, 164] and end-stage heart failure [162, 165, 166]. Myocarditis is an extreme example of how limited acute IL-6 signaling is protective for viral clearance but the chronic, long term exposure of the heart to IL-6 contributes to pathology and loss of cardiac function and remodeling. This dissertation will demonstrate the specific mechanisms by which IL-6 signaling drives these different outcomes.

1.8 Figures and Tables

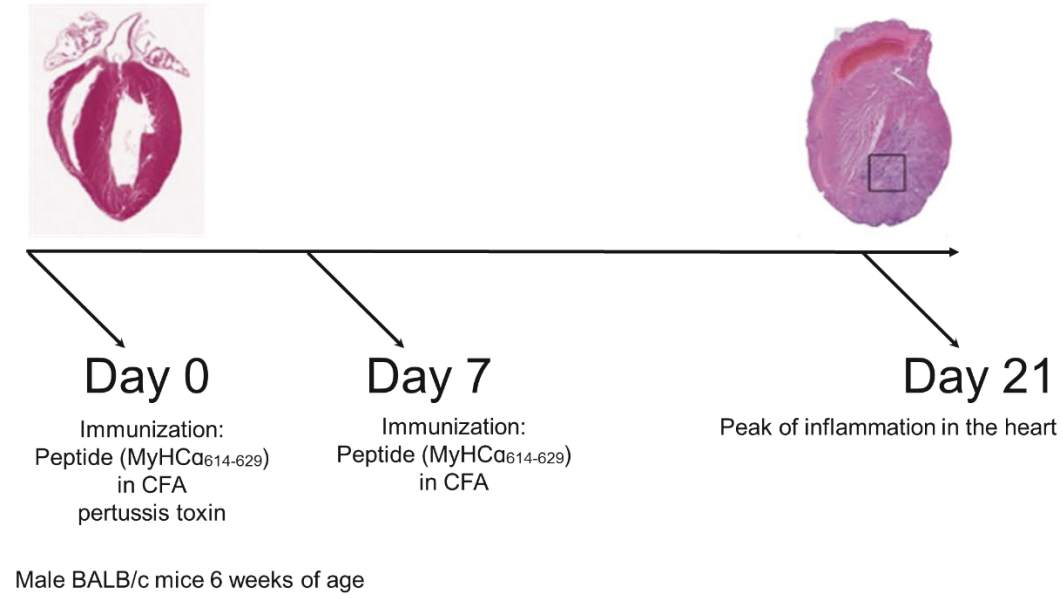

Figure 1.1. Induction of experimental autoimmune myocarditis (EAM)

To induce EAM, male BALB/c mice at 6-8 weeks of age, are immunized the myocarditogenic peptide of cardiac myosin heavy chain, MyHC**α**614-629 (Ac-SLKLMATLFSTYASAD) emulsified in CFA. On days 0 and 7, mice received an axillary subcutaneous immunization of 100 **μ**g of MyHC**α**614-629 peptide emulsified in CFA supplemented to 5 mg/mL of heat-killed *Mycobacterium tuberculosis* strain H37Ra. On day 0, mice additionally received 500 ng of pertussis toxin intraperitoneally. On day 10 following immunization immune cells begin to infiltrate the myocardium. Day 21 is peak of inflammation, when EAM is assessed by flow cytometry or histology.

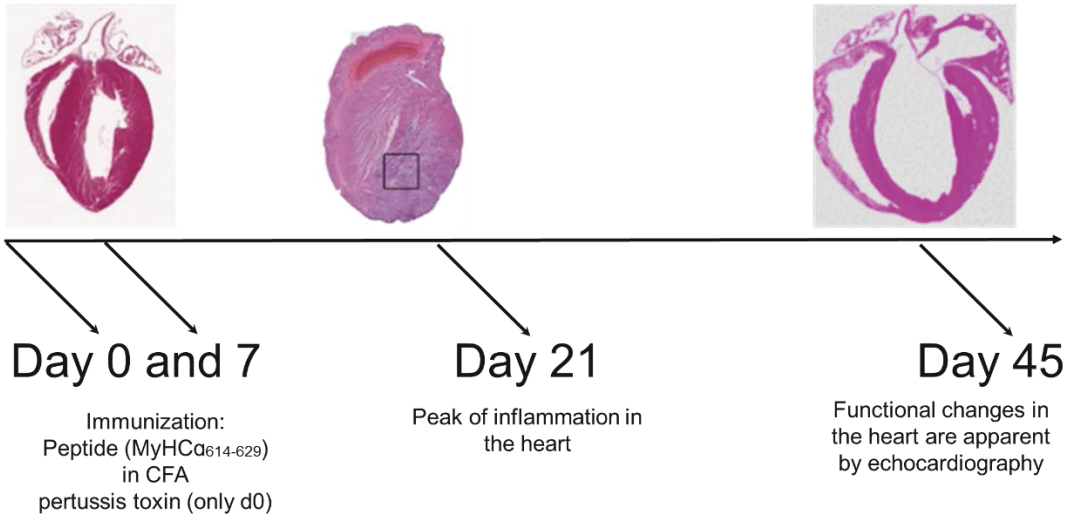

Figure 1.2. Induction of dilated cardiomyopathy

To induce DCM, male BALB/c mice at 6-8 weeks of age, are immunized the myocarditogenic peptide of cardiac myosin heavy chain, MyHC**α**614-629 (Ac-SLKLMATLFSTYASAD) emulsified in CFA. On days 0 and 7, mice received an axillary subcutaneous immunization of 100 **µ**g of MyHC**α**614-629 peptide emulsified in CFA supplemented to 5 mg/mL of heat-killed Mycobacterium tuberculosis strain H37Ra. On day 0, mice additionally received 500 ng of pertussis toxin intraperitoneally. On day 10 following immunization immune cells begin to infiltrate the myocardium. Day 21 is peak of inflammation and EAM. By day 35, heart function begins to decrease as measured by echocardiography. By day 45 hearts are enlarged and DCM is apparent by echocardiography or histology.

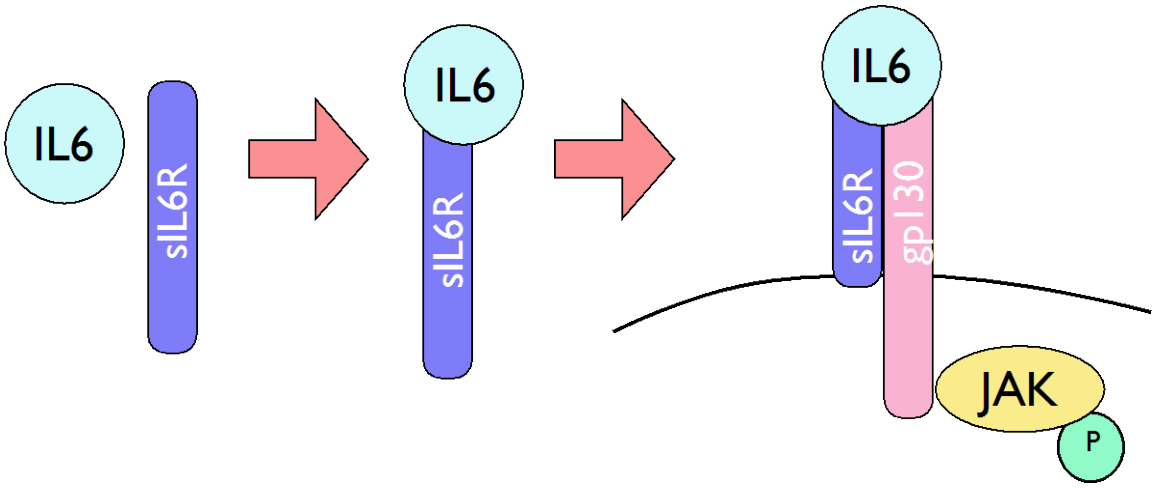

Figure 1.3. IL-6 trans-signaling

IL-6 has the ability to signal to cells that lack the membrane-bound IL-6R. IL-6 can bind with the soluble-IL-6R, which in turn will bind with membrane-bound gp130 to form a signaling complex. This soluble-receptor signaling is termed 'trans'-signaling as opposed to the membrane-bound IL-6R signaling, termed 'classical' IL-6 signaling.

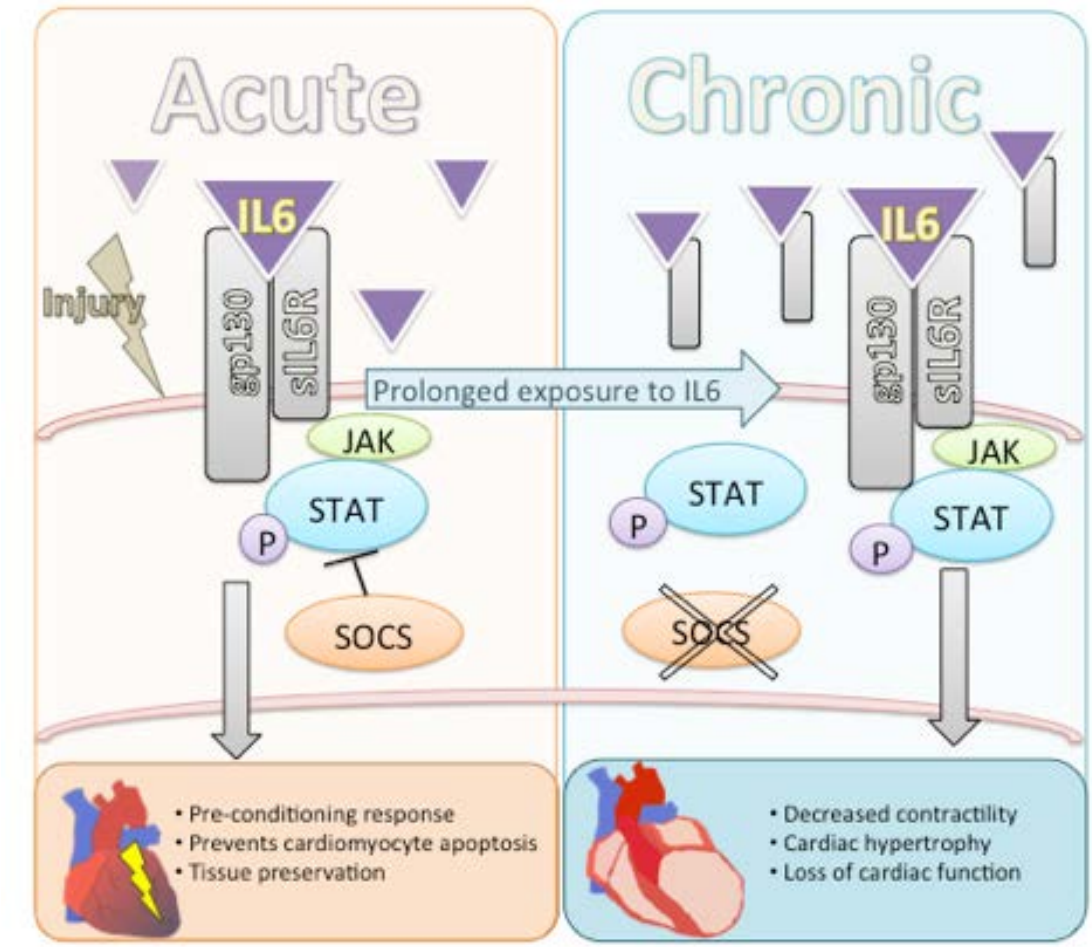

Figure 1.4. Acute and Chronic IL6 signaling in the myocyte

The figure depicts transition from acute, protective IL6 signaling, to chronic, pathogenic IL6 signaling on the cardiomyocyte. In the acute phase (on the left), IL6 preserves cardiac tissue by inducing an anti-apoptotic program in the myocyte and triggers the pre-conditioning response. When IL6 signaling continues chronically, these protective responses become pathogenic and induce depressed myocyte function (on the right). There is decreased contractility, hypertrophic genes are turned and LV enlargement occurs.

Table 1.1 Subtypes of Human Myocarditis

Subtypes of Immune-cell infiltration during Myocarditis in Human		
Infiltration Type	**Likely etiology**	**Outcome**
Giant-cells	**Autoimmune**	**Heart failure**
Eosinophils	**Acute coronary syndrome, cardiogenic shock, hypersensitivity, autoimmune**	**Rapid heart failure and sudden death**
Lymphocytic	**Parastic, viral, fungal, hypersensitivity, autoimmune**	**Resolution, heart failure**
Mixed	**Idiopathic, parastic, viral, fungal, hypersensitivity, autoimmune**	**Resolution, heart failure**

Table 1.2. The dual role of IL-6 in human studies

	Beneficial	**Harmful**
Acute signaling		
Elevated IL-6 serum levels in patients with myocarditis	Limits viral infection [160]	Associated with DCM [163]
Elevated IL-6 serum levels following myocardial infarction	Limits infarct size [148, 149]	Associated with HF and LVF[167]
Chronic signaling		
Long-term elevated IL-6 serum levels		Two fold Increased life-time risk of CHD [4]
Long-term elevated sIL-6R serum levels		Associated with severe RA [118, 119]
Polymorphism in IL-6R leading to elevated IL-6R protein expression in sera		Increased susceptibility to systemic juvenile idiopathic arthritis (SJIA) [98] Predictive of adverse outcomes in CVD [63]
Decreased myocardial SOCS3 protein expression		Found in myocardium of patients with DCM [154]

Table 1.3. The dual role of IL-6 in mouse model studies

	Beneficial	**Harmful**
Acute signaling		
Increased myocyte production of IL-6	Anti-apoptotic Protective against oxidative stress Pre-conditioning response [148, 149]	Depressed basal contractility Hypertrophic genes turned on Depressed b-adrenergic response LV enlargement [145, 151, 152]
IL-6KO mice in experimental myocarditis	Resistant to experimental autoimmune myocarditis [128]	Increased susceptibility to viral-induced myocarditis mouse models [160]
Chronic signaling		
Myocyte-specific gp130 loss	No IL-6 signaling [125]	Susceptible to myocyte apoptosis in pressure-overload models [158]
Cardiac-specific increase in STAT3 expression	Increased IL-6-family responsiveness [124]	Mice develop hypertrophy without stimuli [157]
IL-6KO mice	Resistant to experimentally induced RA [96], colitis [87], EAE [97], EAM [98], autoimmune kidney disease [99]	Susceptible to infections [168, 169]

Chapter II: Interleukin 6 is required for the induction of experimental autoimmune myocarditis

2.1 Introduction

The precise role IL-6 plays in the induction of EAM is unknown. Earlier studies have shown that IL-6 is required for disease induction, as IL-6KO mice do not develop EAM. However, why IL-6 is required remains unclear. The objective of these experiments was to define the role of IL-6 in EAM. We hypothesized that IL-6 was required for the initial induction of EAM based on preliminary data showing that IL-6 levels were significantly elevated following immunization. The aims of this research were to first define the timeframe in which IL-6 was required to induce disease and second, to describe the mechanism of action of IL-6 in EAM induction.

2.2 Results

Mice treated with anti-IL-6-receptor antibodies are protected from EAM

In order to study the role that IL-6 plays in the pathogenesis of EAM in the standard mouse model, we employed an anti-IL-6-receptor blocking antibody (Figure2.1A-E). Wild-type (WT) BALB/c mice were treated with an anti-IL-6R antibody or isotype control and then immunized with the MyHC**α**614-629 peptide emulsified in CFA. At 21 days post immunization, cardiac histopathology analysis revealed that mice receiving the anti-IL-6R antibody were protected from EAM, whereas IgG2a treated controls developed EAM (Figure 2.1A-B). Flow cytometric analysis demonstrated that there were significantly increased numbers of CD45+ leukocytes in the hearts of control

compared to anti-IL-6R treated mice (Figure 2.1C). Anti-IL-6R treatment raised the systemic circulating levels of IL-6 (Figure 2.1D), but IL-6R downstream signaling, as measured by the levels of pSTAT3 in heart homogenates at day 21, was significantly reduced (Figure 2.1E) confirming the efficiency of the IL-6R blocking antibody.

Systemic IL-6 levels increase following immunization with CFA

To isolate the timeframe in which IL-6 is required during EAM, systemic IL-6 levels were profiled during the course of disease as well as in response to CFA specifically. WT BALB/c mice were immunized with MyHC**α**614-629 peptide emulsified in CFA at day 0 and 7 and blood was drawn on the days 0, 3, 10, 15, 21 (Figure 2.2A). Over the course of disease, IL-6 was maintained at elevated levels in the serum. Although IL-6 levels decreased in the serum after day 10, serum levels were significantly elevated compared with baseline values (Figure 2.2A). In order to distinguish the IL-6 response to CFA versus CFA + peptide in the emulsion, mice were immunized either with CFA alone (Mock) or CFA with the MyHC**α**614-629 peptide on day 0. On day 3 post-immunization, there was no significant difference between the IL-6 response to CFA alone and CFA + peptide indicating that, at this early time point, the IL-6 increase is independent of peptide presence in the emulsion (Figure 2.2B). Therefore, the IL-6 increase in the inductive phase of the response does not require the presence of the myosin antigen.

Recombinant IL-6 treatment partially restores susceptibility to EAM in IFA immunized mice

CFA is required for the induction of EAM. IFA induces an antibody response without inducing infiltration into the heart and subsequent lesions and disease. In order to ascertain whether the difference between a pathogenic versus inert response to immunization was related to the production of IL-6, we immunized mice with IFA or CFA with or without recombinant IL-6. WT BALB/c mice were immunized with MyHCα614-629 peptide emulsified in either IFA or CFA at day 0 and 7 (Figure 2.3). In addition to the immunization, mice were either injected with 50 ng of recombinant IL-6 IV at the time of immunization (Figure 2.3). Mice were then sacrificed 21 days post-immunization. Histopathology of mouse hearts showed that mice immunized with IFA alone were protected from heart inflammation, whereas mice immunized with CFA developed heart inflammation (Figure 2.3). In contrast, mice immunized with IFA and treated with recombinant IL-6 at the day 0 immunization developed infiltration in the heart. This finding demonstrates that recombinant IL-6 treatment restores partial susceptibility to EAM in otherwise EAM-resistant mice. Therefore, IL-6 is partially responsible for the difference between a pathogenic and an inert response in the heart in response to immunization.

IL-6 is only required for the initial response to immunization in order to induce EAM

IL-6 is required for the development of EAM (Figure 2.1) IL-6 is also important for the response to adjuvant in inducing pathogenic responses against the heart (Figure 2.3). We then wanted to identify the critical window in which IL-6 is absolutely required for disease induction. It has been reported that IL-6KO mice are completely resistant to EAM [170]. To identify the precise role IL-6 plays in the development of EAM, we treated IL-6KO mice with PBS or recombinant IL-6 in order to compare their disease development with WT mice. WT BALB/c or IL-6KO BALB/c mice were immunized with MyHCα614-629 peptide emulsified in CFA at day 0 and 7. On days -1,0,1,2 mice were treated with either PBS or 50 ng recombinant IL-6 IV (Figure 2.4A). Histopathology of mouse hearts showed that IL-6KO mice receiving recombinant IL-6 developed heart inflammation comparable to WT mice (Figure 2.4B). Therefore, we have established that IL-6 during the inductive phase is sufficient to drive EAM.

IL-6 leads to differential DC profiles in the draining lymph node following immunization

In order to more precisely define the role IL-6 plays in response to immunization, we studied the DC response in the lymph nodes draining the immunization site. BALB/c mice were immunized in the hind limb at day 0 and sacrificed 3 days post-immunization. Flow cytometric analysis showed that mice treated with anti-IL-6R antibodies had significantly lower levels of CD11c+ DCs in the draining lymph nodes of the sites of immunization

(Figure 2.5A). These DCs had lower expression of maturity marker MHCII expression but similar levels of CD8+ expression compared to untreated mice (Figure 2B and data not shown). Therefore, IL-6 appears to be important for the DC response in the draining lymph nodes that is associated with the induction of EAM.

IL-6 induces DC migration to the draining lymph node through DC CCR7+ expression and LN CCL21 expression

To further identify whether IL-6 induced DC maturity or trafficking, we investigated the trafficking markers of the DCs and the draining lymph nodes. BALB/c mice were immunized in the hind limb and sacrificed 3 days post-immunization. The draining lymph node of the immunization site was collected. Lymph node cytokine and chemokine levels were interrogated by ELISA and Multiplex cytokine bead array. DC populations were interrogated by flow cytometry. Interestingly, the levels of LN trafficking marker, CCR7, showed a decrease in the anti-IL6R treated animals (Figure 2.6A). We also investigated the ligand of CCR7, CCL21 expression in the draining lymph node. Using the same experimental design, we isolated the draining lymph nodes and homogenized the whole lymph node. Mice treated with anti-IL-6R antibodies had lower expression of CCL21 (Figure 2.6B). Cytokine and chemokine production in the draining lymph node showed that there were few statistically significant changes, however, there was a trend that DC-

related trafficking markers were decreased in mice treated with anti-IL6R antibodies (Figure 2.7).

Together, these experiments illustrate that IL-6 is required for DC trafficking to the draining lymph node through the upregulation of CCR7 on the DC as well as increased expression of CCL21 by cells of the draining lymph node (Figure 2.6A-B).

2.3 Conclusions

In these experiments, we have altered the systemic levels of IL-6 during EAM induction in WT BALB/c in order to demonstrate the requirement of IL-6 in EAM induction. Using anti-IL-6R antibodies we have shown that IL-6 is necessary for EAM induction. Further, using IL-6KO mice and recombinant IL-6 treatment, we have determined that the necessity for IL-6 is only during the days surrounding the initial CFA immunization. It has previously been shown that IL-6KO mice do not develop disease [170]. We have clarified this finding, demonstrating that the requirement for IL-6 is not throughout the course of EAM, but rather specifically for the immune response to immunization. Additionally, we have elucidated the role IL-6 plays in response to adjuvant. It is known that IFA immunization does not induce organ-specific autoimmune disease in the mouse, and CFA is required for disease induction [171]. In EAM, IFA induces an auto-antibody response without inducing a pathogenic T cell response leading to heart lesions. We have demonstrated that one of the driving immunologic responses to adjuvant is the production of IL-6. Mice treated with recombinant IL-6 on the day of IFA immunization developed a low but detectable level of heart infiltration not previously seen under these challenge conditions.

To determine the mechanism by which IL-6 exerts its effects in the development of an immune response to adjuvant, we used antibody treatment to block IL-6R signaling. On days -3 and day 0 of immunization we treated

WT BALB/c mice either with isotype control antibodies or antibodies directed against the IL-6R. This established mice that either had IL-6 signaling abilities, or did not, with the additional benefit of mice that biologically developed with IL-6 competence as compared with IL-6KO mice. Three days following immunization we interrogated the immune response by multiple methods. Cellular populations in the draining lymph node of the immunization site were analyzed by flow cytometry. Through this extensive characterization of the IL-6-induced immune response to adjuvant, we demonstrated that anti-IL-6R treatment resulted in fewer DCs in the draining lymph node, with lower expression of maturation marker MHCII and trafficking marker CCR7. We also demonstrated that anti-IL-6R treatment lowered the lymph node expression of inflammatory DC chemokine CCL21. Other changes in chemokine and cytokine production in the draining lymph node were mild, but supported the induction of mediators of inflammatory trafficking of cells to the lymph node. Thus, we have shown that in response to immunization, IL-6 induces DC CCR7-CCL21 mediated trafficking to the draining lymph node. This difference in DCs in the draining lymph node is also accompanied by upregulation of maturity marker MHCII by the DCs.

Through these experiments we have elucidated fundamental mechanisms by which IL-6 induces innate response to adjuvant. IL-6 is required for initial DC responses that ultimately lead to the difference between an inert

immunological response without IL-6, versus a robust immunological response, with IL-6.

2.4 Materials and Methods

Mice

WT BALB/cJ and IL-6-/- BALB/c mice were purchased from the Jackson Laboratory. All mice were maintained in the Johns Hopkins University School of Medicine specific-pathogen free vivarium. Experiments were conducted on 6-8 week old male mice, in compliance with the Animal Welfare Act and the principles set forth in the Guide for the Care and Use of Laboratory Animals. All methods and protocols performed were approved by the Animal Care and Use Committee of The Johns Hopkins University.

Induction of EAM

To induce EAM, we employed the myocarditogenic peptide of cardiac myosin heavy chain, MyHC**α**614-629 (Ac-SLKLMATLFSTYASAD). The peptide was commercially synthesized by fMOC chemistry and purified to a minimum of 90% by HPLC (Genscript). On days 0 and 7, mice received an axillary subcutaneous immunization of 100 **μ**g of MyHC**α**614-629 peptide emulsified in either complete Freund's adjuvant (CFA) (Sigma) supplemented to 5 mg/mL of heat-killed *Mycobacterium tuberculosis* strain H37Ra (Difco) or incomplete Freund's adjuvant (IFA) (Sigma). On day 0, mice additionally received 500 ng of pertussis toxin intraperitoneally. (List Biologicals).

Anti-IL-6R Treatment

Wild-type (WT) BALB/c mice were divided into 2 groups of 8 mice each; treatment with an anti-IL-6R antibody (BioXcell) or isotype control, IgG2b (BioXcell). Antibody treatment was given IV at day-7 (1 mg) and day 0 (0.5 mg) of the immunization protocol. These mice were then immunized with the MyHC**α**614-629 peptide emulsified in CFA and sacrificed 21 days post-immunization.

Assessment of EAM Histopathology

Mouse hearts were evaluated for the development of EAM on day 21. Heart tissues were fixed in SafeFix solution (Fisher Scientific). Tissues were embedded in a paraffin block in a longitudinal orientation and 5 **μ**m serial sections were cut and stained with hematoxylin and eosin (H&E) (HistoServ, Gaithersburg, MD). Myocarditis severity was evaluated by visually estimating the area of the myocardium infiltrated with hematopoietic cells using the published scoring system: grade 0, no inflammation; grade 1, less than 10% of the heart section is involved; grade 2, 10-25%; grade 3, 25-50%; grade 4, 50-75%; grade 5, more than 75%. Grading was performed by grading sections per heart by two independent, blinded investigators and the values were expressed as an average.

Flow Cytometry Analysis and FACS Isolation of Heart Infiltrating Cells

To prepare the cellular infiltrating populations for flow cytometry analysis, single cell suspensions were made from mouse hearts. Hearts were perfused for 3 min with 1x phosphate buffered saline (PBS) + 0.5% FBS, and digested in GentleMACS C Tubes according to manufacturer's instructions (Miltenyi Biotec). Viability was determined by LIVE/DEAD staining according to manufacturer's instructions (Life Techonologies). Cells were blocked with **α**CD16/32 (eBiosciences), and surface markers were stained with fluorochrome-conjugated mAbs (eBioscience, BD Pharmingen, BioLegend). Samples were acquired on the LSR II cytometer running FACSDiva 6 (BD Immunocytometry). Data were analyzed with FlowJo 7.6 (Treestar Software).

ELISA

Quantitative sandwich ELISA for cell culture supernatants were determined by colorimetric ELISA kits according to manufacturers'recommended protocols (R&D Systems and Sigma).

Multiplex

Quantitative Multiplex for tissue homogenates were determined by bead-based Multiplex kits according to manufacturers'recommended protocols (Milltenyi).

Statistics

Normally distributed data were analyzed by two-tailed Student's t-test (for data containing two groups) or one-way ANOVA followed by Tukey's post-test (for data with multiple groups). EAM severity scores were analyzed by Mann-Whitney U test (up to two groups). Values of $p < 0.05$ were considered statistically significant.

2.5 Figures

Figure 2.1 Mice treated with anti-IL-6-receptor antibodies are protected from EAM

EAM was induced in WT BALB/c mice treated with istotype control IgG antibodies or anti-IL-6-receptor antibodies on days 0 and 7. Mice were sacrificed 21 days post-immunization. Myocarditis severity was assessed by cardiac histopathology and heart-infiltrating cells were analyzed by flow cytometry.

(A-B) Myocarditis severity was assessed by cardiac histopathological scoring using H&E staining. Data points represent individual mice. Bars represent mean. Data are analyzed by Mann-Whitney U test. Data are representative of 3 independent experiments.

(C) Absolute number of intracardiac vital CD45+ leukocytes in heart. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

(D) Systemic IL-6 levels measured from serum at day 21-post immunization by ELISA. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

(E) Phosphorylated STAT-3 levels measured from total heart homogenate at day 21-post immunization by ELISA. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

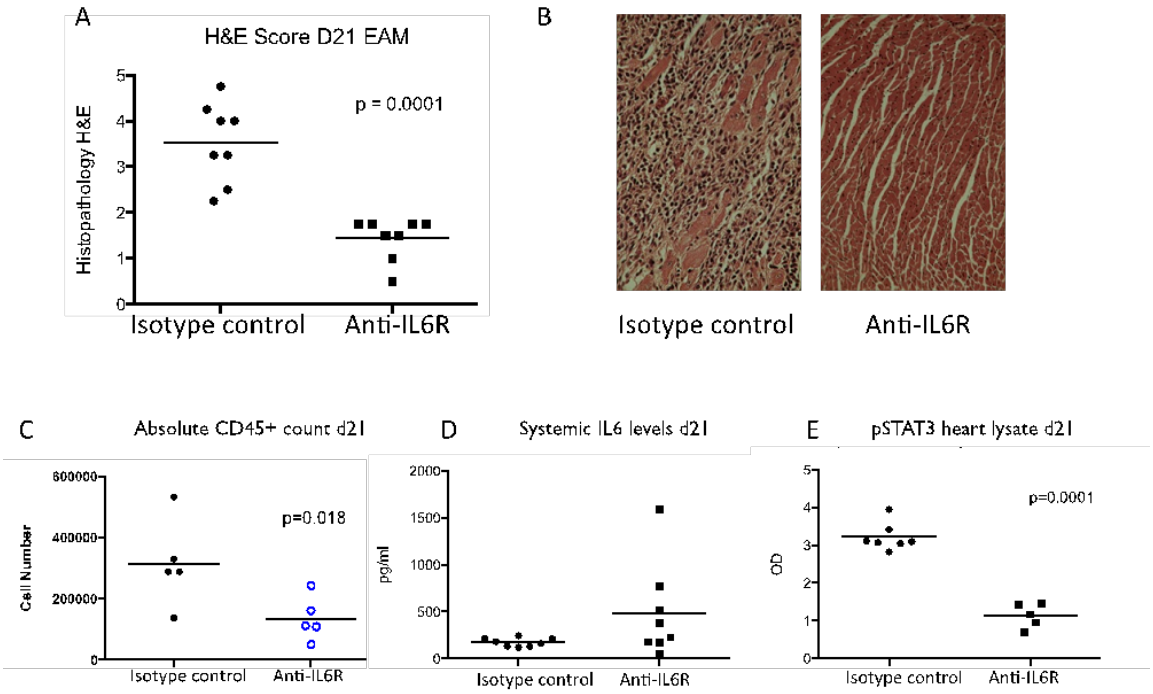

Figure 2.1 Mice treated with anti-IL-6-receptor antibodies are protected from EAM

Figure 2.2. Systemic IL-6 levels increase following immunization with CFA

EAM was induced in WT BALB/c mice and serum levels of IL-6 were profiled in response to immunization.

(A) EAM was induced in WT BALB/c mice. Blood was drawn on days 0, 3, 10, 15, and 21 in order to assess serum IL-6 levels by ELISA. Data points represent individual mice. Bars represent mean. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$.

(B) Mice were immunized with either CFA alone, without peptide (mock) or CFA containing the myosin-derived peptide (myosin). Blood was drawn on day 3 in order to assess serum IL-6 levels by ELISA. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

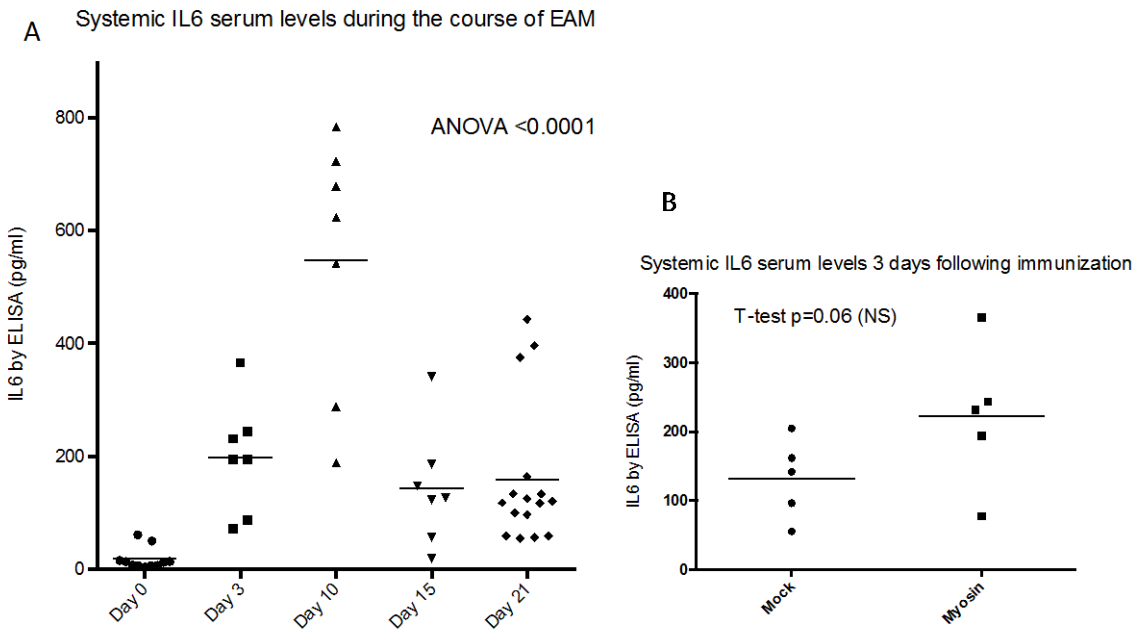

Figure 2.2. Systemic IL-6 levels increase following immunization with CFA

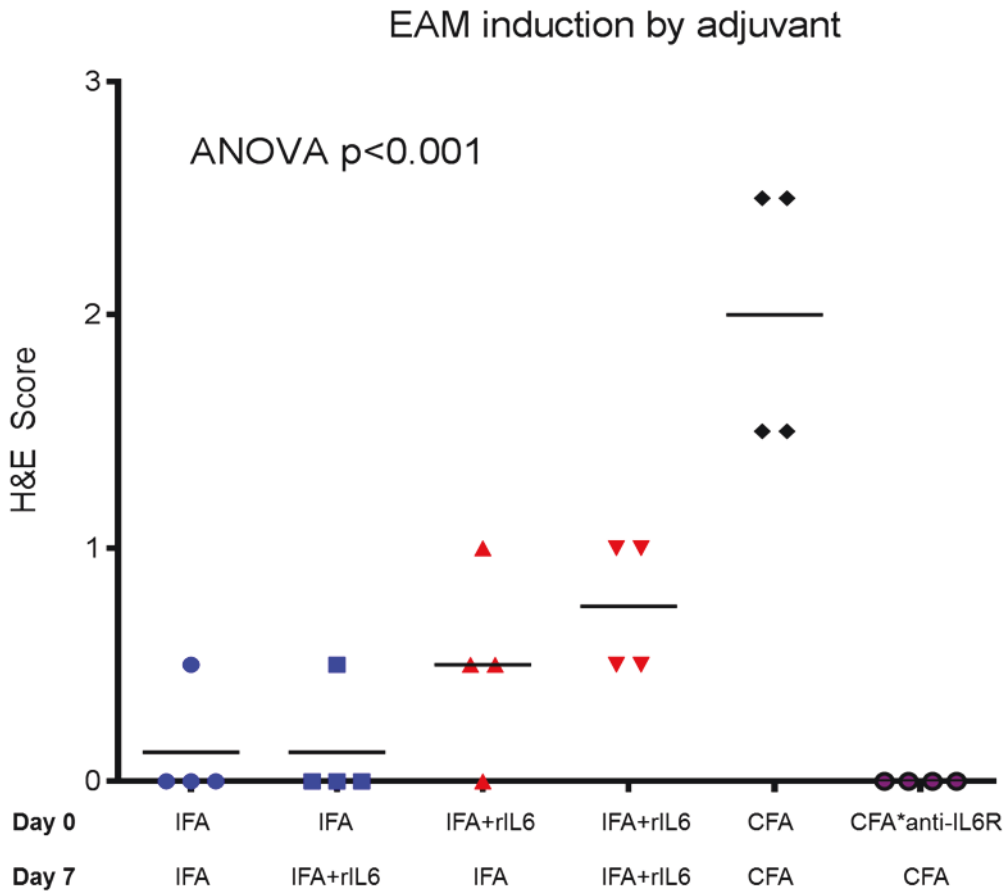

Figure 2.3. Recombinant IL-6 treatment restores susceptibility to EAM in IFA immunized mice

EAM was induced in WT BALB/c mice using varying immunizations. Mice were immunized with IFA or CFA, alone or with recombinant IL6 treatment on the day indicated (x-axis). Mice were sacrificed 21 days post-immunization. EAM was assessed by histopathology.

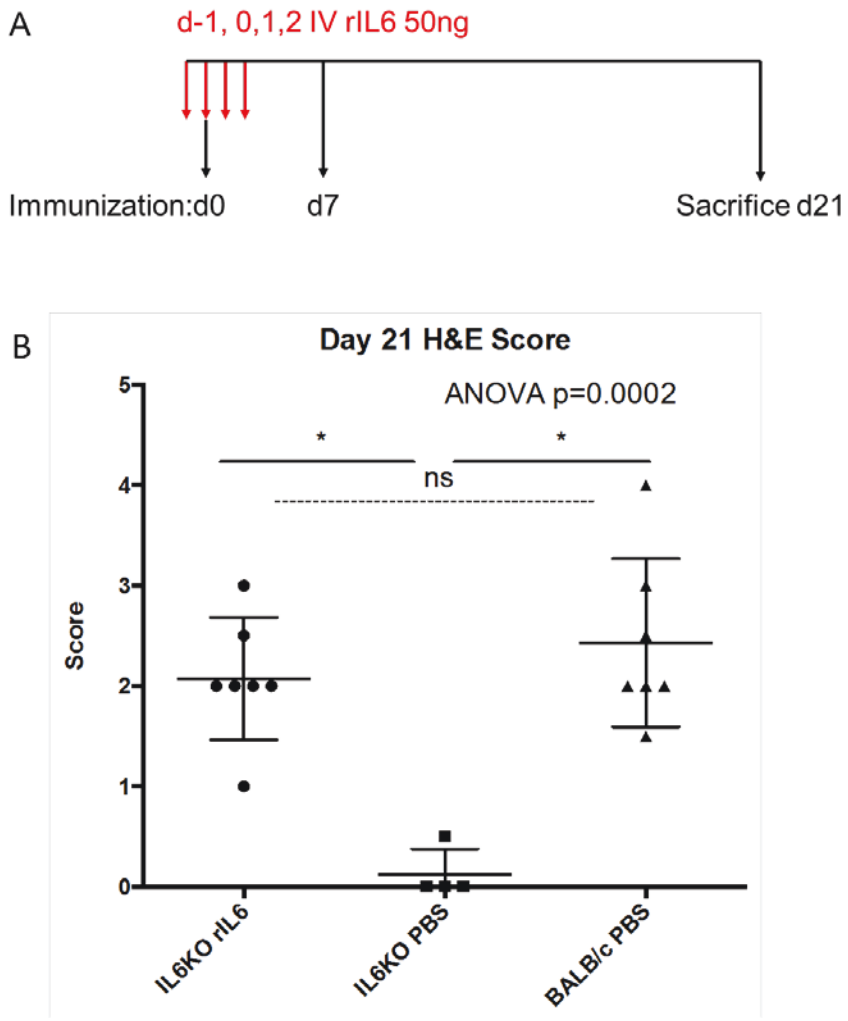

Figure 2.4. IL-6 is only required for the initial response to immunization in order to induce EAM

EAM was induced in WT BALB/c mice or IL-6KO mice treated with or without recombinant IL-6. Mice were sacrificed 21 days post-immunization. EAM was assessed by histopathology.

(A) Schedule of recombinant IL-6 treatment and EAM immunization.

(B) Histopathological scoring of EAM in mice hearts scored using H&E staining. Data points represent individual mice. Bars represent mean. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$.

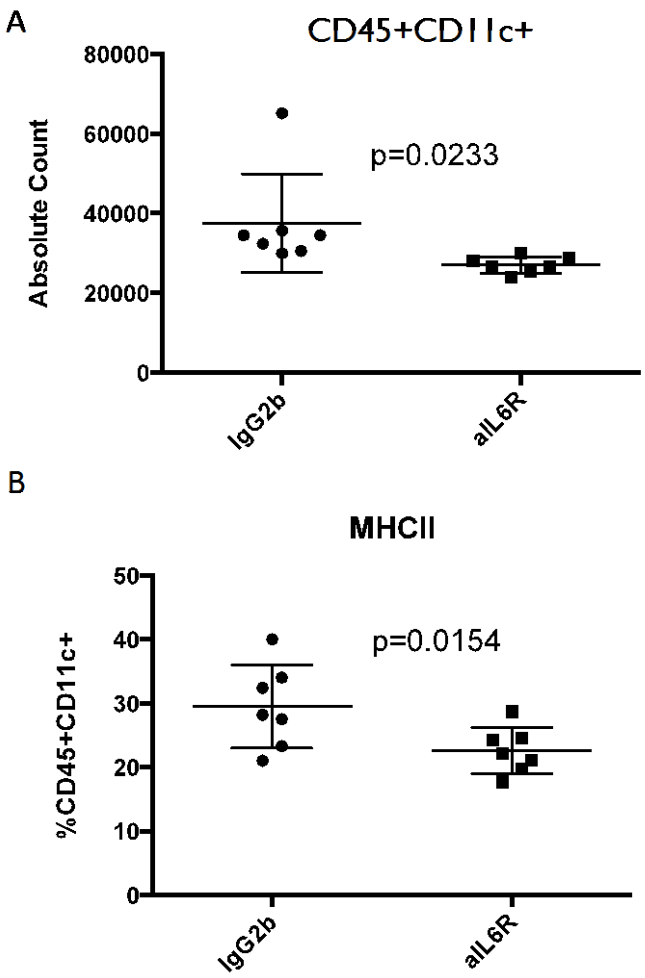

Figure 2.5. IL-6 leads to differential DC profiles in the draining lymph node following immunization

EAM was induced in WT BALB/c mice treated with istotype control IgG2b antibodies or anti-IL-6-receptor antibodies on days -3 and 0. Mice were sacrificed 3 days post-immunization.

(A-B) Response to immunization was assessed by interrogating immune cells in the draining lymph node of the immunization site by flow cytometry. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. Data are representative of 3 independent experiments.

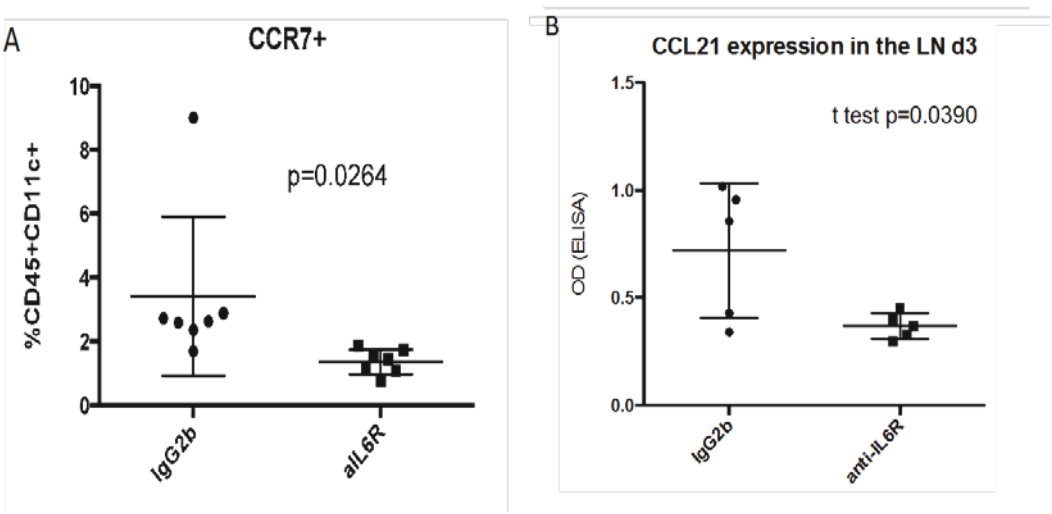

Figure 2.6. IL-6 induces DC migration to the draining lymph node through DC CCR7+ expression and LN CCL21 expression

EAM was induced in WT BALB/c mice treated with istotype control IgG2b antibodies or anti-IL-6-receptor antibodies on days -3 and 0. Mice were sacrificed 3 days post-immunization.

(A) Response to immunization was assessed by interrogating immune cells in the draining lymph node of the immunization site by flow cytometry. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. Data are representative of 3 independent experiments.

(B) Total lymph node homogenates were assessed for CCL21 levels by ELISA. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

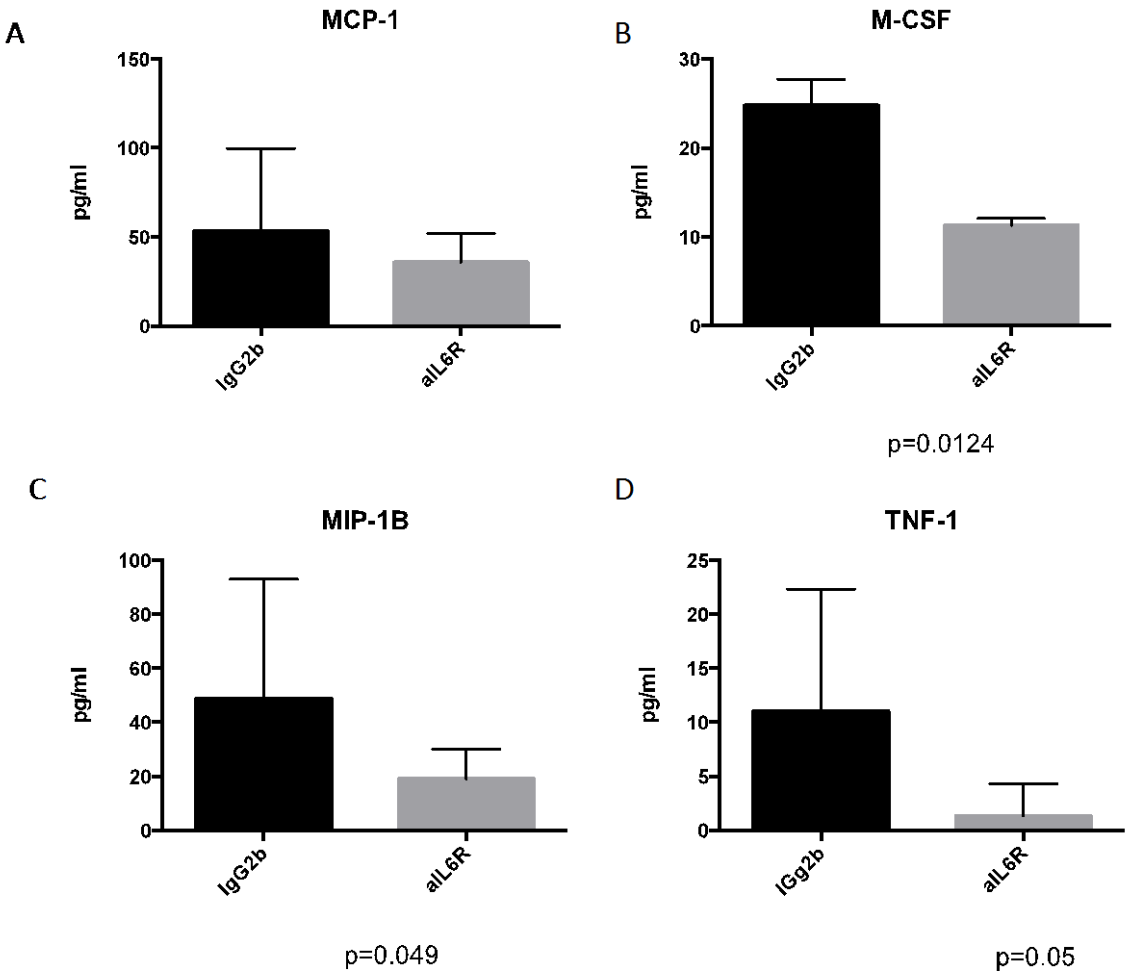

Figure 2.7. Cytokine and chemokine production in the immunization site draining lymph node 3 days post immunization

EAM was induced in WT BALB/c mice treated with istotype control IgG2b antibodies or anti-IL-6-receptor antibodies on days -3 and 0. Mice were sacrificed 3 days post-immunization.

(A-D) Total lymph node homogenates were assessed for protein levels of analytes by micro-bead array. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

Chapter III: Interleukin 6 signaling on cardiac myocytes drives inflammatory dilated cardiomyopathy

3.1 Introduction

In Chapter 2 we demonstrated that IL-6 is required to induce EAM during the induction phase of disease. Following disease induction the role of IL-6 is undefined. It is known that in patients with heart failure increased circulating levels of IL-6 correlate with poor outcomes. We thus hypothesized that following EAM, during the progression to DCM, increased IL-6 levels would lead to decreased heart function. The aims of this research were to define the role of IL-6 in the pathogenesis of DCM and describe the mechanism of action of IL-6.

3.2 Results

Circulating IL-6 levels during EAM correlate with heart failure progression

It is known that in humans, increased circulating levels of IL-6 correlate with heart failure [4]. It has recently been shown that high circulating IL-6 is an independent risk factor for heart disease [92-94]. To investigate the correlation between circulating IL-6 during EAM to heart failure in DCM we interrogated levels of IL-6 in sera of mice with EAM. WT BALB/c mice were immunized with MyHCα614-629 peptide emulsified in CFA at day 0 and 7 and blood was drawn on day 21 of EAM (Figure 3.1). Elevated serum IL-6 levels at the peak of EAM at day 21, correlate with negative heart outcomes by echocardiography on day 45 (Figure 3.1). This mouse data corresponds to

the profile seen in human patients, validating the use of this model to interrogate the role of IL-6 in DCM development.

IL-6 is required for the development of dilated cardiomyopathy

In order to test whether IL-6 was necessary for DCM development, we treated mice with anti-IL-6R antibodies and followed these mice through to heart failure. We have shown that this antibody blocks the development of EAM when given at the time of immunization (Figure 2.1). Therefore to study the progression to DCM, we treated the mice with anti-IL-6R after the induction of EAM was already established (Figure 3.2). WT BALB/c mice were immunized with MyHCα614-629 peptide emulsified in CFA at day 0 and 7 and separated into 3 different groups. Mice were treated IV on days 14, with 1mg and 21, with 0.5mg with either PBS alone, control isotype IgG2b antibodies or anti-IL-6R antibodies. Heart function was then evaluated by echocardiography on day 50 and following, the mice were euthanized for necropsy. Mice treated with anti-IL-6R antibodies retained normal heart function and were protected from ventricular dilation as compared with PBS or isotype treated mice (Figure 3.2). Thus, we established that the IL-6R signaling pathway is required for the development of DCM.

Increasing circulating levels of IL-6 in BALB/c mice worsens heart function without altering fibrosis in the heart

In order to test whether increasing IL-6 levels in IL-6-competent mice could drive worsened heart failure, we increased the circulating levels of IL-6 in WT BALB/c mice during DCM. To do this we employed hydrodynamic gene delivery of a plasmid containing an IL-6 insert under the control of a ubiquitous promoter. Following IV injection of the plasmid containing the IL-6 insert, circulating levels of IL-6 were elevated in the serum for up to 3 weeks (Figure 3.3). Thus, this method provides an efficient tool for increasing the circulating levels of IL-6 in the mouse. WT BALB/c mice were immunized with MyHCα614-629 peptide emulsified in CFA at day 0 and 7 and separated into 2 different groups. Mice were injected IV on day 14 with 2 mL of saline containing 50 ug of plasmid either with no insert or the IL-6 insert. Heart function was then evaluated by echocardiography on day 45 and following, the mice were euthanized for necropsy. Mice treated with the plasmid containing the IL-6 insert developed worse heart function as compared with mice treated with the plasmid without the insert.(Figure 3.4A-B). It is important to note that both groups developed DCM, however the IL-6 plasmid group had increased severity of DCM (Figure 3.4A-B). Thus, we established that further increases in IL-6 can drive worse heart function in DCM. However, despite differing levels of heart function, both groups demonstrated similar levels of fibrosis in the heart (Figure 3.4C). These results were confirmed by analyzing fibrosis in earlier time points when effectors of fibrosis are normally altered in the model (Figure 3.5). This is

particularly interesting because it suggests that the effects of IL-6 in the heart are particularly important on the cardiac myocyte rather than cells responsible for cardiac fibrosis, which includes fibroblasts.

In order to confirm that the IL-6 plasmid treatment was directly affecting heart function, the plasmid treatment of mice was also interrogated at day 21 following day 14 IV hydrodynamic gene delivery. IL-6 plasmid treatment did not significantly alter the number of CD45+ cells infiltrating the heart (Figure 3.6), raising the possibility that the DCM phenotype was a result of IL-6 signaling on the cardiac resident cells, and not through changes to immune populations.

IL-6 signaling through the cardiomyocyte is required for progression to dilated cardiomyopathy

IL-6 worsens cardiac function without affecting the fibrosis in the heart (Figure 3.4- 3.6). Blocking IL-6 signaling in the heart also protects the heart from heart failure (Figure 3.2). These two results taken together suggest that IL-6 may assert its pathogenic effects by acting on the cardiac myocyte. In order to test this hypothesis we studied the course of DCM in mice that do not have the ability to signal through IL-6 on the cardiac myocyte alone. IL-6 signaling on the cardiac myocyte is through trans-signaling as the myocyte does not express the surface bound IL-6R required for classical signaling [107]. We obtained mice from the laboratory of Kirk Knowlton, GP CKO mice,

that lack myocyte IL-6 signaling by knocking out the gp130 receptor on the myocyte [158]. GP CKO mice have a myocyte-restricted knockout of the *gp130* gene by employing a Cre-lox strategy. The *gp130* floxed allele mice contain 2 loxP sites in the transmembrane domain of gp130. The Cre mice delivered restriction to the myocyte using a knock-in strategy, utilizing Cre coding sequences into the genomic locus of the myosin light chain 2v (*MLC2v*) gene. Thus, GP CKO mice have normal IL-6 responses in every cell and tissue, including fibroblasts, except for the cardiac myocyte [158, 172, 173]. These mice provide a powerful tool to test the role of IL-6 in heart failure during EAM progression to DCM. Naïve GP CKO have normal heart function (Figure 3.7) and are susceptible to EAM (Figure 3.8). Additionally, during EAM, BALB/c and GP CKO mice have similar number of heart infiltrating CD45+ and CD4+ T cells (Figure 3.9). We have not observed any differences in proportion of IFNY+CD4+, IL17A+CD4+, and GM-CSF+CD4+ T cells in the hearts of GP CKO mice compared with WT animals during EAM (Figure 3.9). However, interestingly, GP CKO mice are completely protected from DCM (Figure 3.10A-C). WT BALB/c mice or GP CKO were immunized with MyHC**α**614-629 peptide emulsified in CFA at day 0 and 7. Heart function was assessed by echocardiography on day 45 and showed GP CKO mice were protected from heart failure and dilation (Figure 3.10B-C). Histopathology of mouse hearts showed that GP CKO were also protected from cardiac fibrosis, whereas WT BALB/c controls showed profound fibrosis

(Figure 3.10A). Heart fibrosis was assessed for collagen deposition in the heart by Masson's trichrome staining.

In order to confirm that CFA immunization alone in GP CKO mice did not contribute to heart failure, GP CKO mice were 'mock' immunized, according to the same schedule and experiment, with CFA alone (without peptide). These mice did not develop EAM (Figure 3.8) or DCM (Figure 3.10A-C). Therefore, using GP CKO mice, we have shown that IL-6 signaling is required on the cardiac myocyte for the development of DCM (Figure 3.10A-C).

IL-6 induces IL-15 production by cardiomyocytes

Having established that IL-6 signaling to cardiac myocytes is essential in driving cardiac failure during DCM, we sought to identify mechanism by which IL-6 exerts its effects. We isolated primary cardiac myocytes from adult WT mice and adult GP CKO mice and stimulated them with IL-6 alone (classical signaling), IL-6 and soluble-IL-6R together (trans-signaling) or IL-1b (a known myocyte-responsive cytokine). After 24 hours the supernatant and the cardiac myocytes were collected. Supernatants were analyzed using a bead-based Multiplex array for protein concentration (Milltenyi). Most of the cytokine and chemokines examined were comparable between conditions (Figure 3.11). However, WT myocytes produced IL-15 in response to IL-6+soluble-IL-6R as opposed to stimulated GP CKO myocytes (Figure 3.12B-

3.13A). This response was not seen with IL-6 alone, demonstrating myocytes require signaling in trans (Figure 3.12). This result was confirmed in heart homogenates from WT and GP CKO mice, comparing naïve to immunized mice sacrifice at day 21 of EAM (Figure 3.13B). It is known that IL-15 is made by myocytes in skeletal as well as cardiac tissue and is an autocrine cytokine that protects cells from apoptosis. Therefore, this result uncovers a fascinating role of IL-6 in the basic biology of myocyte signaling, and suggests IL-15 autocrine signaling may contribute to chronic IL-6-mediated alteration of myocyte function.

IL-6 signaling to the cardiomyocyte alters the profile of heart-function-related proteins associated with depressed myocyte contraction and heart dilation

Chronic IL-6 signaling reduces the basal contractility of the myocyte, induces a hypertrophic response and ultimately results in loss of myocyte function [156, 172, 173]. Therefore, we investigated whether the mechanism of IL-6 in the pathogenesis of EAM-associated DCM was depressing the contractile capability of the myocyte. IL-6 was shown to down-regulate proteins required for the pumping of calcium, called SERCA (sarcoendoplasmic reticulum calcium transport ATPase) proteins, in myocytes *in vitro* [174]. Additionally, *in vitro*, IL-6 up regulates atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which mediate loss of myocyte function [175]. Therefore, we examined the levels of these proteins from the hearts of

WT BALB/c mice compared with GP CKO mice during DCM by Western Blot (Figure 3.14A-C).

SERCA proteins are calcium-ATPases that reside in the sarcoplasmic reticulum (SR) of the myocyte that transfer calcium from the cytosol to the SR for muscular excitation and contraction [176]. SERCA2 is the isoform found in the cardiac myocyte specifically [177, 178]. Decreased SERCA2 expression correlates to depressed myocyte contraction [179]. Hearts from WT BALB/c mice during DCM have lower levels of SERCA2 than GP CKO mice (Figure 3.14A). This suggests that IL-6 signaling during DCM depresses cardiac myocyte function as GP CKO mice, which are protected from DCM, do not display this phenotype (Figure 3.10).

Additionally, WT BALB/c mice have higher levels of ANP and BNP proteins (Figure 3.14B-C). ANP, atrial natriuretic peptide, is released from myocytes in response to mechanical stress [180]. BNP, brain natriuretic peptide, is released primarily from the heart [181]. The release of ANP and BNP is increased in patients with heart failure [182]. ANP and BNP levels increase in response to changes in heart filing pressures [183]. The increase seen in ANP and BNP levels in the hearts of WT BALB/c mice compared with GP CKO mice is further evidence that IL-6 signaling on the cardiac myocyte is driving DCM through the loss of myocyte function (Figure 3.14A-C). Therefore, during EAM, IL-6 signals to the cardiac myocyte causing loss of function and driving heart failure in DCM.

3.3 Conclusions

In patients with heart failure, increases in circulating levels of IL-6 correlate with negative heart outcomes [105]. This association has been extensively studied and it was recently shown that high levels of circulating IL-6 are an independent risk factor for heart disease in healthy people [91]. Patients with DCM have been shown to have multiple changes in IL-6 signaling [154]. DCM patients have higher levels of IL-6 in the circulation [162, 163] but have also been shown experimentally to have changes associated with IL-6R signaling as well [154]. For these reasons we examined the role of IL-6 in EAM-associated DCM. Similar to patients, we demonstrated that higher concentrations of IL-6 in the serum of mice during EAM predict poor heart outcomes during the DCM phase of disease. This result led us to investigate the mechanism by which IL-6 exerts its pathogenic effects.

We have shown that IL-6 is required for the development of EAM. However, once EAM has been established, IL-6 levels remained chronically elevated compared to baseline IL-6 concentrations in the serum. Therefore we examined what role this proinflammatory cytokine was playing in the development of heart failure. Using an anti-IL-6R antibody treatment during EAM, we demonstrated that IL-6 is required for the development of DCM. Mice treated with an anti-IL-6R antibody at days 14 and 21 of EAM were completely protected from DCM, with normal both heart function by

echocardiography and no development of fibrosis by hydroxyproline as compared with PBS or isotype treated mice.

Knowing that high levels of circulating IL-6 are associated with heart failure, we investigated whether we could drive disease progression further by increasing IL-6 levels. Using IL-6-competent BALB/c mice, we utilized hydrodynamic gene delivery to in order to artificially increase circulating IL-6. Mice that had increased IL-6 levels above the normal course of IL-6 induction during disease had hearts which were more dilated and had worse heart function compared with untreated mice. However, the loss of heart function was not accompanied by worsened fibrosis in the heart. Both treated and untreated mice developed fibrosis, but treated mice, with worse heart function, did not have increased fibrosis above the normal course of disease. We further confirmed these results by interrogating mediators of fibrosis earlier in disease. We demonstrated that various mediators of fibrosis, including matrix metalloproteinases (MMPs), tissue inhibitors of matrix metalloproteinases (TIMPs) and collagen levels were unchanged between groups during EAM. In order to directly relate the heart dilation phenotype to myocytes we interrogated the heart infiltrating immune populations of treated and untreated mice during EAM showing that infiltrating populations were not significantly altered during hydrodynamic gene delivery. Thus, we determined that the decreased heart function associated with increased IL-6 levels was due to direct effects on the heart. This was

particularly interesting as the evidence suggested that the pathogenic effects of IL-6 were specifically attributed to function alone and not fibrosis.

In order to address the IL-6 differences in function versus fibrosis we examined the cell types of the heart responsible for each. IL-6 signaling on cardiac fibroblasts is known to produce mediators of fibrosis whereas heart function is attributed to cardiac myocytes. Thus, we investigated the role of IL-6 signaling on cardiac myocytes. In order to isolate the signaling on myocytes, we employed genetically altered mice. GP CKO mice have a cre-lox knock-in deletion of the gp130 cellular receptor on myocytes alone [158]. These mice have myocytes that cannot respond to IL-6 because the cells lack the signaling receptor gp130 required [158]. GP CKO mice are developmentally normal, with normal heart function during homeostasis [158, 172, 173]. GP CKO mice have heart function comparable with WT BALB/c mice (Supplemental Figure 5). Thus, GP CKO mice are a powerful tool in which to study the IL-6 effects on the myocyte.

Using GP CKO mice, we demonstrated that IL-6 signaling on the cardiac myocyte is required for the development of DCM. We demonstrated that GP CKO develop WT BALB/c-comparable EAM, but are completely protected from the progression to DCM. Thus, IL-6 signaling on the cardiac myocyte is required for the development of DCM.

In order to determine the mechanism by which IL-6 signaling to the cardiac myocyte causes DCM we interrogated the cardiac cells *in vitro* and the hearts *in vivo*, between genotypes. We isolated cardiac myocytes from naïve WT BALB/c and GP CKO hearts and treated them *in vitro* with cytokines. Myocytes make few cytokines and chemokines. Many of the targets we interrogated were found to be unchanged, however, interestingly we showed that myocytes make IL-15 in response to IL-6+solubleIL-6R signaling. IL-15 is an autocrine hormone known to be anti-apoptotic to skeletal muscle [184] which is interesting because IL-6 is known to be anti-apoptotic to cardiac myocytes [185].

IL-15 is known to be produced by muscle cells, in the heart and periphery [186]. In the immune system, IL-15 acts similarly to IL-2, however IL-15 preferentially stimulates CD8+ T cells rather than CD4+ T cells [187]. It is known that EAM is driven by CD4+ T cells, rather than CD8+ T cells, therefore additional IL15 biology was investigated.

Interestingly, IL-15 is known to be an anabolic cytokine in skeletal muscle that protects the cells from apoptosis [184]. IL-6 is known to be anti-apoptotic to the cardiac myocyte [158], suggesting that one of the mechanisms by which IL-6 preserves myocytes from death might be through IL-15 upregulation. Chronic IL-6 signaling on the myocyte is anti-apoptotic, but eventually leads to myocytes with ineffective pumping capacity. These results demonstrated that one of the ways IL-6 may exert anti-apoptotic

effects in the cardiac myocyte may be through IL15 expression. These experiments demonstrated interesting IL-6 basic biology, but did not reveal the specific mechanism of IL-6 pathogenesis on the myocyte.

Chronic IL-6 signaling has been shown to decrease basal contractility of the myocyte, induce a hypertrophic genetic profile in the myocyte and lead to loss of pumping function [145, 153]. Because we have demonstrated that IL-6 acts through the myocyte to drive DCM, we then examined whether the pathogenic effects of IL-6 in DCM were due to direct depression of myocyte function.

To study the function of the cardiac myocyte *in vivo* during DCM we examined the production of myocyte-derived contractile proteins. We interrogated the levels of 3 proteins, known to be made in the myocyte, which are direct correlates to myocyte function. We studied the protein expression, by Western blot, of sarcoendoplasmic reticulum calcium transport ATPase-2 (SERCA2), atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) during DCM. SERCA2 is the heart isoform within SERCA proteins and is required for myocyte excitation and contraction [176, 188]. SERCA expression is decreased in heart failure [189]. Additionally, SERCA gene transfer has been experimentally shown to restore contractile function in myocytes isolated from failing hearts [190]. During the DCM phase after immunization, WT BALB/c mice have lower levels of SERCA2 when compared to immunized GP CKO mice, which are protected from heart

failure. This suggests that IL-6 signaling on the myocyte causes dilation in part due to decreased SERCA2 protein expression, which is known to depress cardiac function [175, 176].

ANP and BNP are two additional proteins made by myocytes [183]. Both have been shown to be increased in failing hearts [181]. ANP and BNP are released by the myocyte in response to mechanical stress [181]. They are produced by failing hearts as a protective measure but are a signal of loss of function [183]. WT BALB/c mice have higher levels of both ANP and BNP in their hearts during DCM compared with GP CKO mice, which are protected from DCM. These results provide additional evidence to the result that IL-6 causes loss of function of the myocyte, as demonstrated by loss of SERCA2 expression. Taken together, the profile of expression of these 3 proteins suggest that the mechanism by which IL-6 signaling on the myocyte leads to DCM is through the direct loss of myocyte function.

Thus, we have shown that IL-6 is required for DCM. Specifically, IL-6 signaling on the cardiac myocyte is required for DCM. Mechanistically, IL-6 signaling on the myocyte results in decreased SERCA expression and increased ANP and BNP expression, together signifying myocyte loss of function. In addition, we have shown that one of the ways in which IL-6 exerts an anti-apoptotic effect on the cardiac myocyte may be through IL15 expression and subsequent autocrine signaling. Therefore IL-6 drives

progression of EAM to DCM by altering proteins associated with decreased myocyte function.

It is known that IL-6 contributes not only to DCM, but to many heart diseases [91, 92]. Our investigation has delineated IL-6 biology on the myocyte that is applicable to many of these diseases. As in many heart diseases, circulating IL-6 levels increase during EAM, and we have investigated the mechanism by which IL-6 drives subsequent heart failure. These results demonstrate that IL-6 signaling on the myocyte drives heart failure, which is particularly interesting for human health because of the mechanics of IL-6 signaling to the myocyte. Because myocytes require IL-6 to signal in trans, due to their lack of the membrane-associated IL-6R necessary for classical signaling, therapeutic targeting of the soluble IL-6R presents the opportunity to affect pathogenic signaling while preserving the other classical signaling functions of IL-6 required systemically. This would be particularly important for heart failure patients requiring long term therapeutics, where classical IL-6 signaling would be required for normal response to insult or injury independent of the pathogenic sIL-6R+IL-6 signaling in heart failure.

3.4 Materials and Methods

Mice

WT BALB/cJ and IL-6-/- BALB/c mice were purchased from the Jackson Laboratory. GP CKO mice were a generous gift from the laboratory of Kirk Knowlton at the University of California, San Diego. All mice were maintained in the Johns Hopkins University School of Medicine specific-pathogen free vivarium. Experiments were conducted on 6-8 week old male mice, in compliance with the Animal Welfare Act and the principles set forth in the Guide for the Care and Use of Laboratory Animals. All methods and protocols performed were approved by the Animal Care and Use Committee of The Johns Hopkins University.

Induction of EAM and DCM

To induce EAM, we employed the myocarditogenic peptide of cardiac myosin heavy chain, MyHC**α**614-629 (Ac-SLKLMATLFSTYASAD). The peptide was commercially synthesized by fMOC chemistry and purified to a minimum of 90% by HPLC (Genscript). On days 0 and 7, mice received an axillary subcutaneous immunization of 100 **μ**g of MyHC**α**614-629 peptide emulsified in either complete Freund's adjuvant (CFA) (Sigma) supplemented to 5 mg/mL of heat-killed Mycobacterium tuberculosis strain H37Ra (Difco) or incomplete Freund's adjuvant (IFA) (Sigma). On day 0, mice additionally received 500 ng of pertussis toxin intraperitoneally. (List Biologicals).

Assessment of EAM Histopathology

Mouse hearts were evaluated for the development of EAM on day 21 or DCM around day 45. Heart tissues were fixed in SafeFix solution (Fisher Scientific). Tissues were embedded in paraffin block longitudinally, and 5 μm serial sections were cut. Staining for EAM at day 21 was with hematoxylin and eosin (H&E), staining for DCM around day 45 was with Masson's trichrome blue (HistoServ, Gaithersburg, MD). Myocarditis severity was evaluated by visually determining the area of the myocardium infiltrated with hematopoietic cells. The area was infiltrated was evaluated using the published scoring system: grade 0, no inflammation; grade 1, less than 10% of the heart section is involved; grade 2, 10-25%; grade 3, 25-50%; grade 4, 50-75%; grade 5, more than 75%. Grading was performed by evaluating 3 sections per heart by two independent, blinded investigators. Values from the 3 section grading and both evaluators were averaged. The same methods were utilized in order to score the area of the heart covered by interstitial fibrosis after staining of sections with Mason's Tricome.

Flow Cytometry Analysis and FACS Isolation of Heart Infiltrating Cells

To prepare the cellular infiltrating populations for flow cytometry analysis, single cell suspensions were made from mouse hearts. Hearts were perfused for 3 min with 1x phosphate buffered saline (PBS) + 0.5% FBS, and digested

in GentleMACS C Tubes according to manufacturer's instructions (Miltenyi Biotec). Viability was determined by LIVE/DEAD staining according to manufacturer's instructions (Life Techonologies). Cells were blocked with **α**CD16/32 (eBiosciences), and surface markers were stained with fluorochrome-conjugated mAbs (eBioscience, BD Pharmingen, BioLegend). Samples were acquired on the LSR II cytometer running FACSDiva 6 (BD Immunocytometry). Data were analyzed with FlowJo 7.6 (Treestar Software).

Echocardiography

Trans-thoracic echocardiography was performed using the Acuson Sequoia C256 ultrasonic imaging system (Siemens). Prior to evaluation, the chest of the mouse was shaved using depilatory cream (Nair). Conscious mice were held in supine position. The heart was imaged in two-dimensional (2-D) mode in the parasternal short axis view. From this mode, an M-mode cursor was positioned perpendicular to the interventricular septum (IVS) and the left ventricular posterior wall (LVPW) at the level of the papillary muscles. From M-mode, the wall thicknesses and chamber dimensions were measured. For each mouse, three to five values for each measurement were obtained and averaged for evaluation. The left ventricular end-diastolic dimension (LVEdD), LV end-systolic dimension (LVEsD), interventricular septal wall thickness at end-diastole (IVSD), and LV posterior wall thickness at end diastole (LVPWTED) were measured from a frozen M-mode tracing. Fractional shortening (FS), ejection fraction (EF) and relative wall thickness

(RWT) were calculated from these parameters as previously described (Baldeviano et al., 2010).

In vivo hydrodynamic gene delivery

Mammalian expression vectors (pORF plasmids) encoding the intronless murine cDNA for IL-6 (pORF-mlL-6) were obtained from Invivogen (San Diego, CA). Plasmids were transformed into competent GT100 *E. coli* bacteria and grown on 250 mL of LB broth containing ampicillin (100 ug/mL). Large-scale preparation of plasmids was performed using the endotoxin-free Maxiprep kit from Qiagen (Valencia, CA). Following quantification by a nanodrop spectrophotometer, 25 ng of plasmid was dissolved in 2 mL of Ringer's lactate solution, and injected into mice by the tail vein. In vivo expression was confirmed by ELISA quantification of IL-6 from sera of mice. Mice injected with pORF-mlL-6 or empty pORF plasmid were immunized with MyHC6i4-629/CFA the next day to induce EAM.

Gel Zymography

MMPs were measured by gel zymography using previously published protocol [191]. Briefly, heart tissue was homogenized in lysis buffer (25 mM Tris-HCI, pH 7.5; 100 mM NaCI; and 1% v/v Nonidet P-40), and cell debris was removed by centrifugation. Total protein was quantified by BCA protein assay kit (Thermo scientific, Rockford, IL). Twenty-five micrograms of total

protein was loaded into a precast 10% Tris-Glycine gel with 0.1% gelatin (Invitrogen). Gels were developed and stained as reported elsewhere [191].

Real-Time Quantitative PCR

Tissue total RNA was extracted in TRIZOL (Life Technologies). cDNA were synthesized with High Capacity cDNA Reverse Transcription Kit (Life Technologies) and amplified with Power SYBR Green Mastermix (Life Technologies) in MyiQ2 thermocycler (Bio-Rad) running iQ5 software (Bio-Rad). Data were analyzed by the 2–ΔΔCt method of Livak, et al., comparing threshold cycles first to *Hprt* expression, then ΔCt of target genes in controls[192].

Hydroxyproline Assay

Heart samples were weighed, homogenized in de-ionized water, and then hydrolyzed in 6N HCl overnight at 120℃ Lysates are transferred and desiccated in 96-well plates, and reconstituted in de-ionized water. After incubation with 50 mM Chloramine T (Sigma), followed by 1M dimethylaminobenzaldehyde (Sigma), the OD values are read at 570 nm. The concentration of hydroxyproline is determined by a 1-100 μg/mL standard curve of hydroxyproline (Sigma) and normalized to starting heart sample mass [193].

Isolation of Primary Adult Mouse Cardiac Myocytes

Hearts were dissected from 6-8 week old male mice pre-treated with heparin, aorta were cannulated, and hearts were perfused with calcium-free perfusion buffer, and digested by type II collagenase (Worthington). Cardiomyocytes were separated from resulting suspensions by their rapid spontaneous precipitation. Isolated cardiomyocytes were cultured in mouse laminin-coated plates or chamber slides and used for experiments after 24 hours.

ELISA

Quantitative sandwich ELISA for cell culture supernatants weredetermined by colorimetric ELISA kits according to manufacturers'recommended protocols (R&D Systems and Sigma).

Multiplex

Quantitative Multiplex for tissue homogenates were determined by bead-based Multiplex kits according to manufacturers' recommended protocols (Milltenyi).

Western Blot

Tissues or cells were collected in RIPA buffer with anti-protease enzymes (Sigma). Total protein was quantified by BCA assay (Thermo Scientific). 20**µ**g of sample was separated on a 10% SDS-PAGE Mini-Protean precast gels (Life Technologies). Bands were transferred to PVDF membrane (vendor). SERCA2 was blotted with mAb clone 2861 (Cell Signaling), BNP was blotted

with mAb clone 19645 (Cell Signaling), ANP was blotted with mAb clone 14348 (Cell Signaling), and **β**-actin was blotted with mAb clone 13E5 (Cell Signaling). HRP-conjugated secondary antibodies (Jackson ImmunoResearch) and Amersham ECL Prime detection system (GE Healthcare) were used to visualize the bands.

Statistics

Normally distributed data were analyzed by two-tailed Student's t-test (for data containing two groups) or one-way ANOVA followed by Tukey's post-test (for data with multiple groups). EAM severity scores were analyzed by Mann-Whitney U test (up to two groups) or Kruskal-Wallis test followed by Dunn's procedure (for more than 2 groups). Values of $p < 0.05$ were considered statistically significant.

3.5 Figures and tables

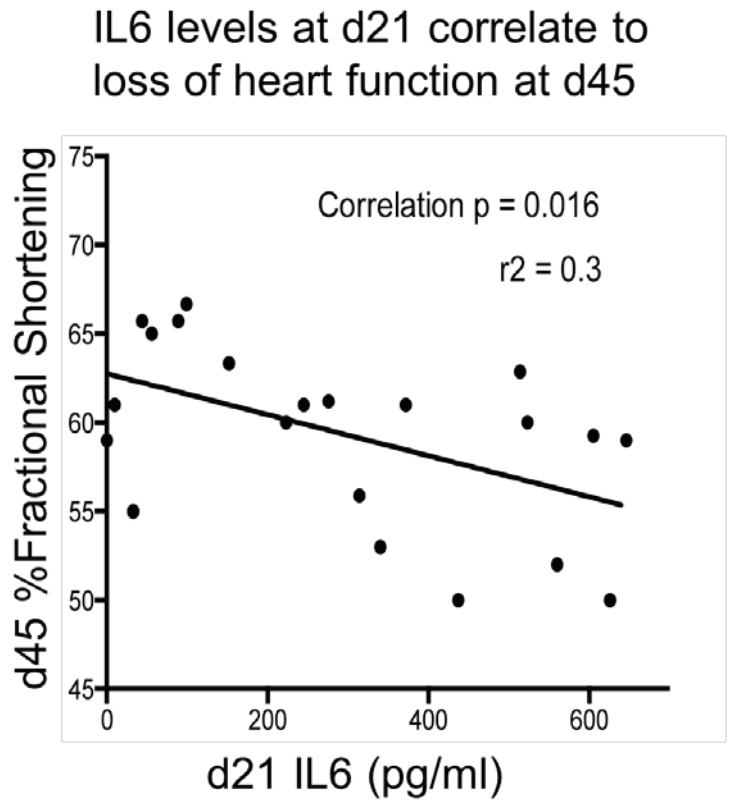

Figure 3.1. Circulating IL-6 levels during EAM correlate with heart failure progression

EAM was induced in WT BALB/c mice and serum levels of IL-6 were profiled at day 21 and then compared to the echocardiography functional outcome at day 45. EAM was induced in WT BALB/c mice. Blood was drawn on day 21 in order to assess serum IL-6 levels by ELISA. The heart function of the mice was assessed at day 45. The levels of IL-6 at day 21 were correlated with the corresponding mouse's echocardiography parameters at day 45 [Fractional shortening (above) as well as ejection fraction (not shown)]. Data points represent individual mice. Data are analyzed by linear regression and correlation analysis.

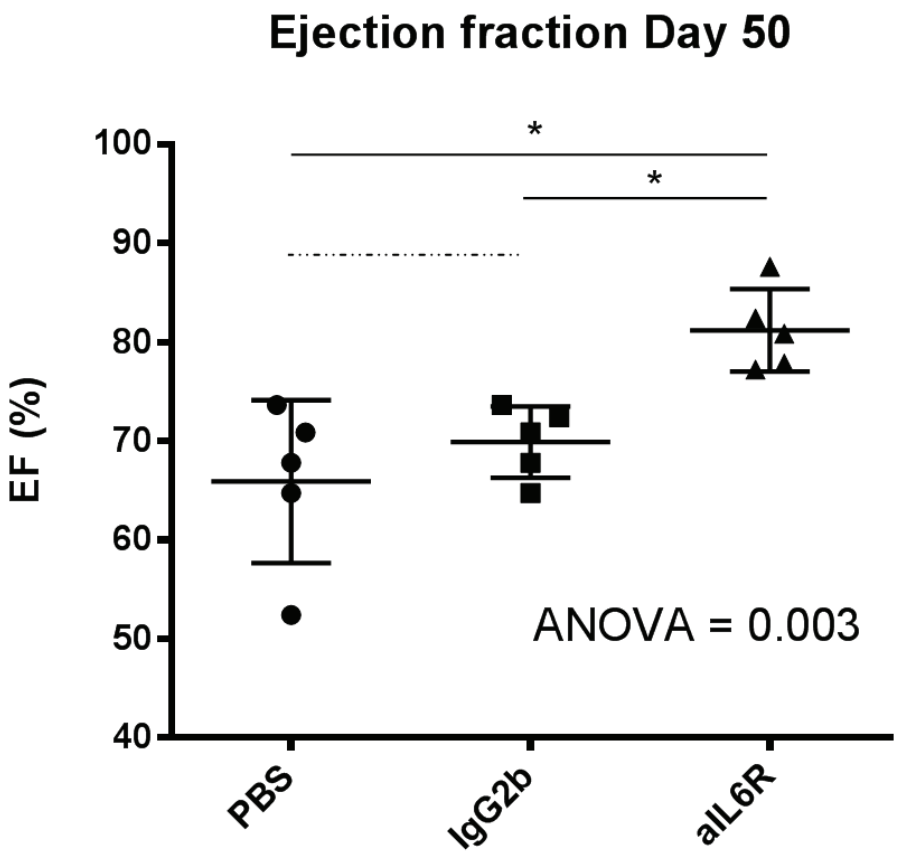

Figure 3.2. IL-6 is required for the development of dilated cardiomyopathy

EAM was induced in WT BALB/c mice treated with PBS, istotype control IgG2b antibodies or anti-IL-6-receptor antibodies on days 14 and 21. DCM was assessed by echocardiography for heart function by measuring ejection fraction (EF) of the heart. Mice were sacrificed 45 days post-immunization. Data points represent individual mice. Bars represent mean and standard deviation. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$. Data are representative of 3 independent experiments.

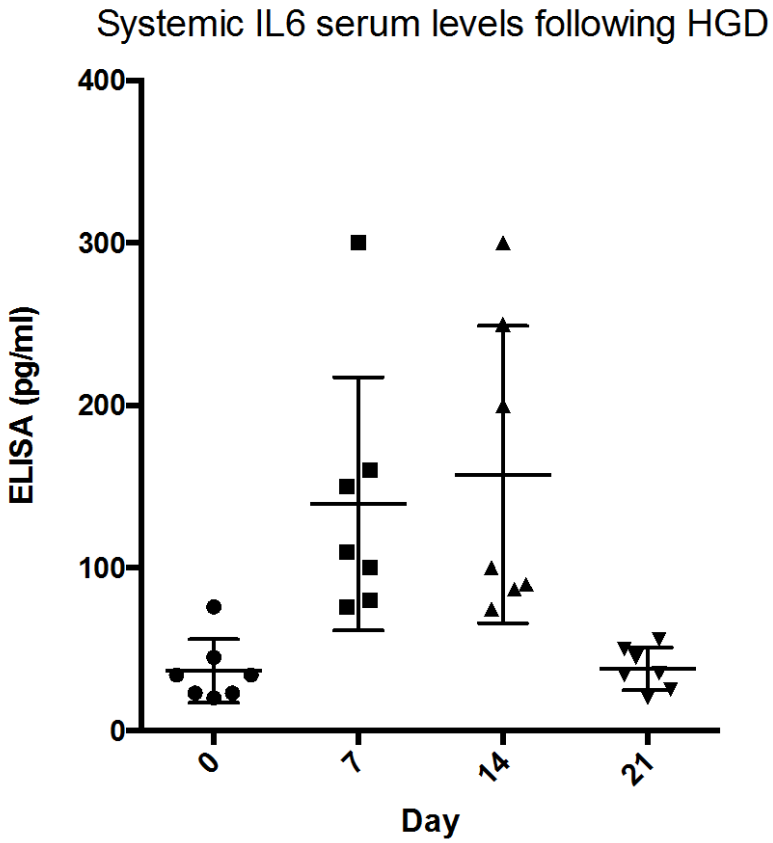

Figure 3.3. Serum levels of IL-6 following hydrodynamic gene delivery of plasmid containing IL-6 insert

Naïve mice were injecting with a plasmid containing an IL-6 insert. Blood was drawn on days 0, 7, 14, and 21 in order to assess serum IL-6 levels by ELISA. Data points represent individual mice. Bars represent mean.

Figure 3.4. Increasing circulating levels of IL-6 in wild-type BALB/c mice worsens heart function without altering fibrosis in the heart

EAM was induced in WT BALB/c mice with HGD of a plasmid with no insert 'pORF-blank' or an IL-6 containing insert 'pORF-IL6' on day 7. Mice were sacrificed 45 days post-immunization. DCM was assessed by echocardiography and collagen deposition in the heart. Data are representative of 3 independent experiments.

(A-B) Echocardiography of mice at day 45. Ejection fraction and left ventricle end diastolic diameters (LVEDD) were measured on awake mice. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

(C) Collagen deposition was assessed by the measurement of hydroxyproline levels in the hearts. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

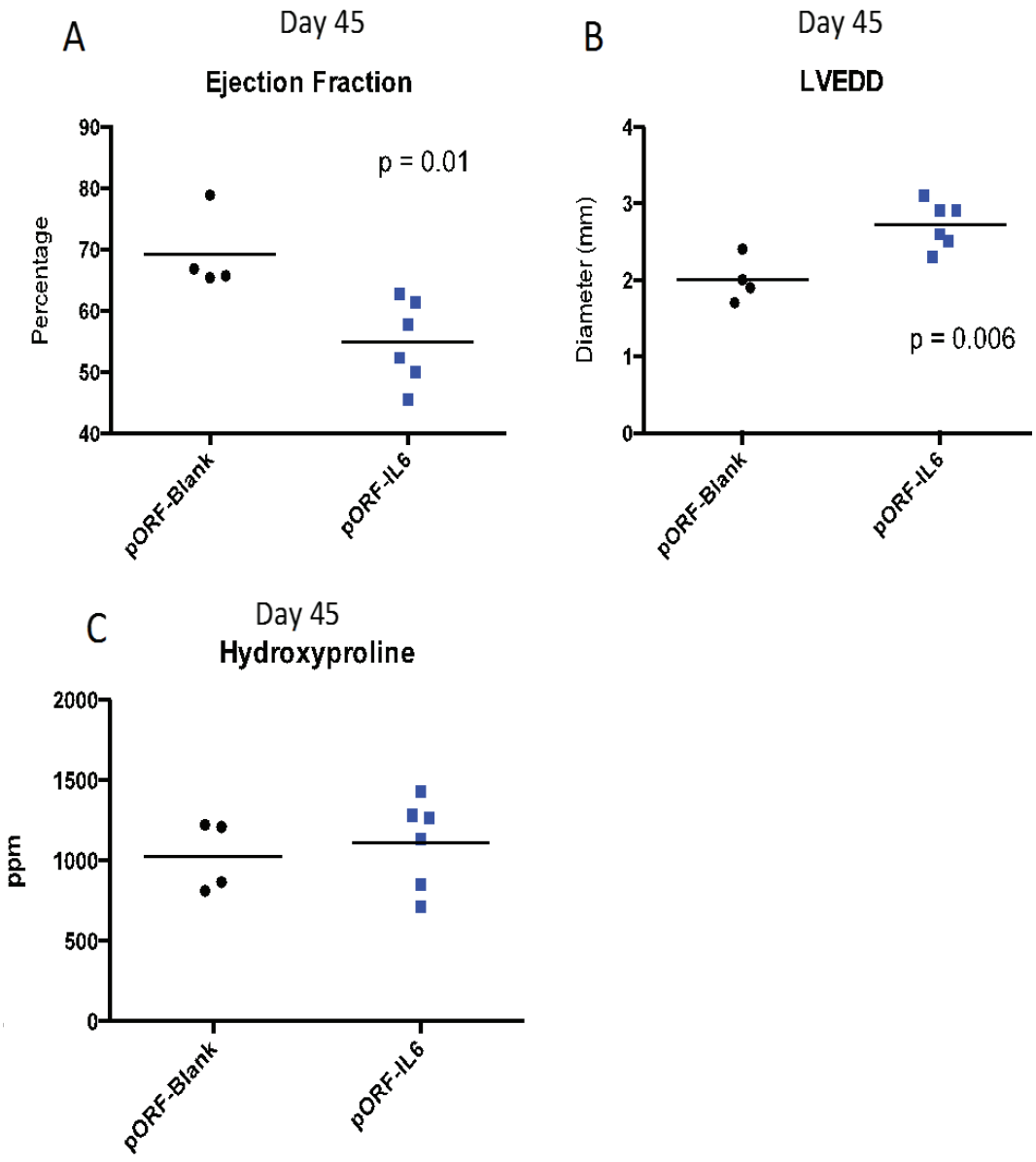

Figure 3.4. Increasing circulating levels of IL-6 in wild-type BALB/c mice worsens heart function without altering fibrosis in the heart

Figure 3.5 Hydrodynamic gene delivery does not alter fibrosis during EAM

EAM was induced in WT BALB/c mice with HGD of a plasmid with no insert 'pORF-blank' or an IL-6 containing insert 'pORF-IL6' on day 7. Mice were sacrificed 14 or 21 days post-immunization. Fibrosis was assessed.

(A) Mice were sacrificed at day 14 and fibrosis was assessed by gel zymography for MMP2 and MMP9 levels. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

(B) Mice were sacrificed at day 21 and fibrosis was assessed by qPCR for mRNA levels of fibrosis associated targets. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test.

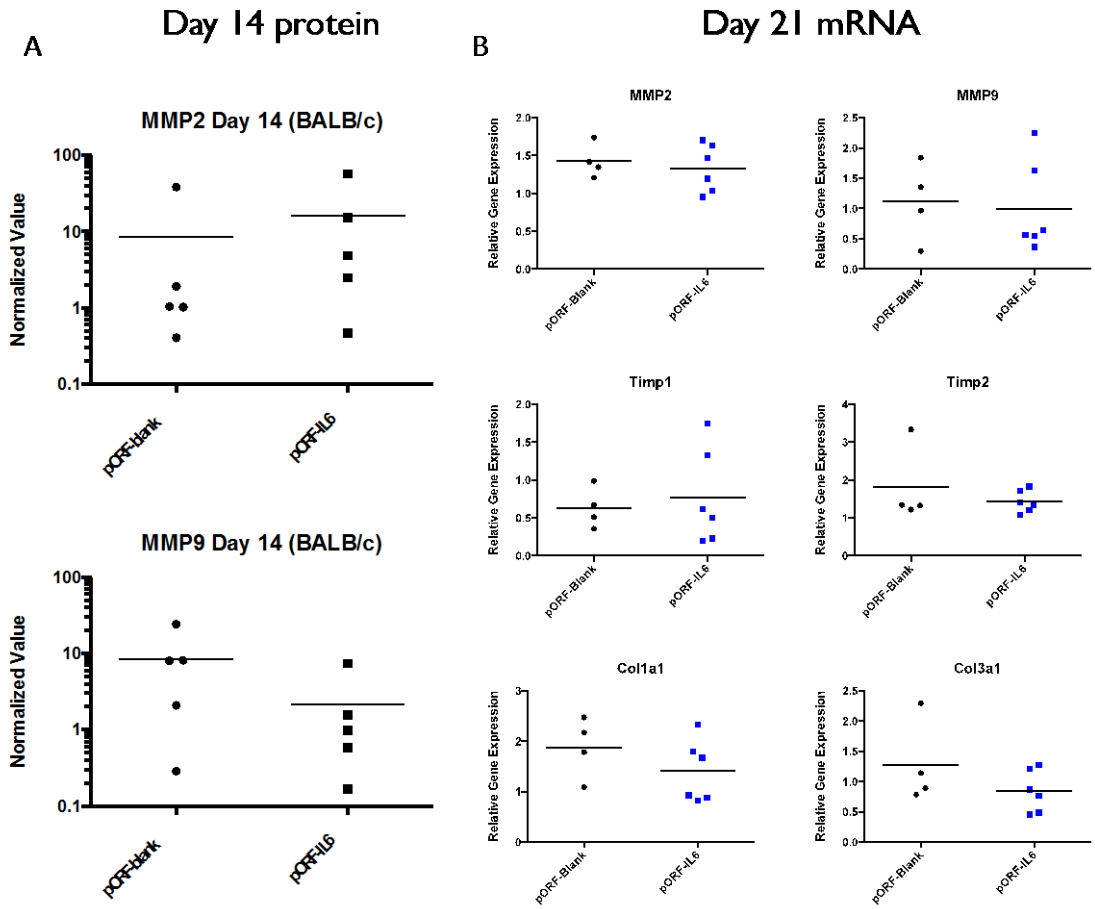

Figure 3.5 Hydrodynamic gene delivery does not alter fibrosis during EAM

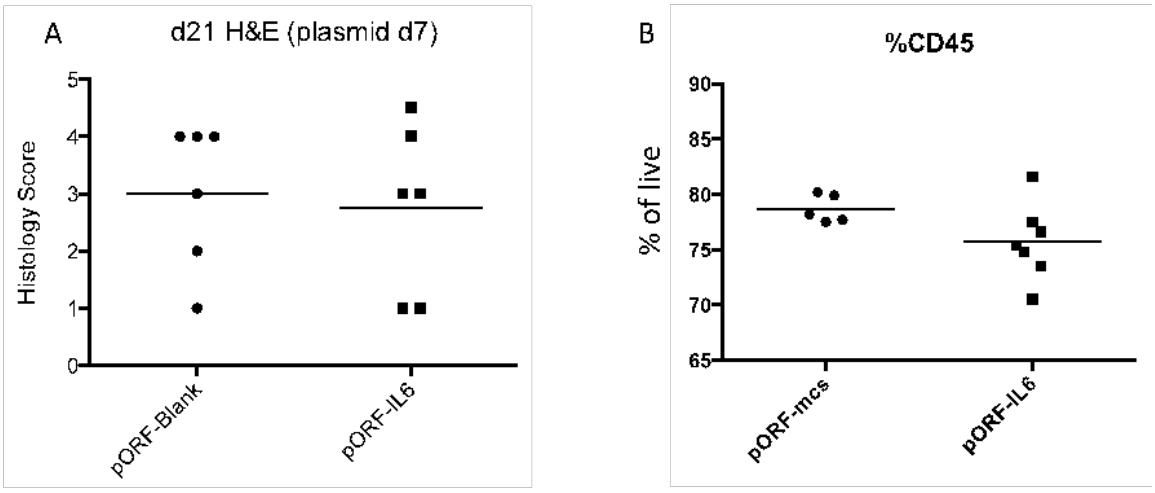

Figure 3.6 Hydrodynamic gene delivery does not alter heart-infiltration populations during EAM

EAM was induced in WT BALB/c mice with HGD of a plasmid with no insert 'pORF-blank' or an IL-6 containing insert 'pORF-IL6' on day 7. Mice were sacrificed 21 days post-immunization. EAM was assessed by histopathology and flow cytometry.

(A) Histopathological determination of EAM. Heart sections were stained with H&E and cellular infiltration into the heart was assessed. (NS)

(B) Total intracardiac CD45+ leukocytes in mice hearts. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. (NS)

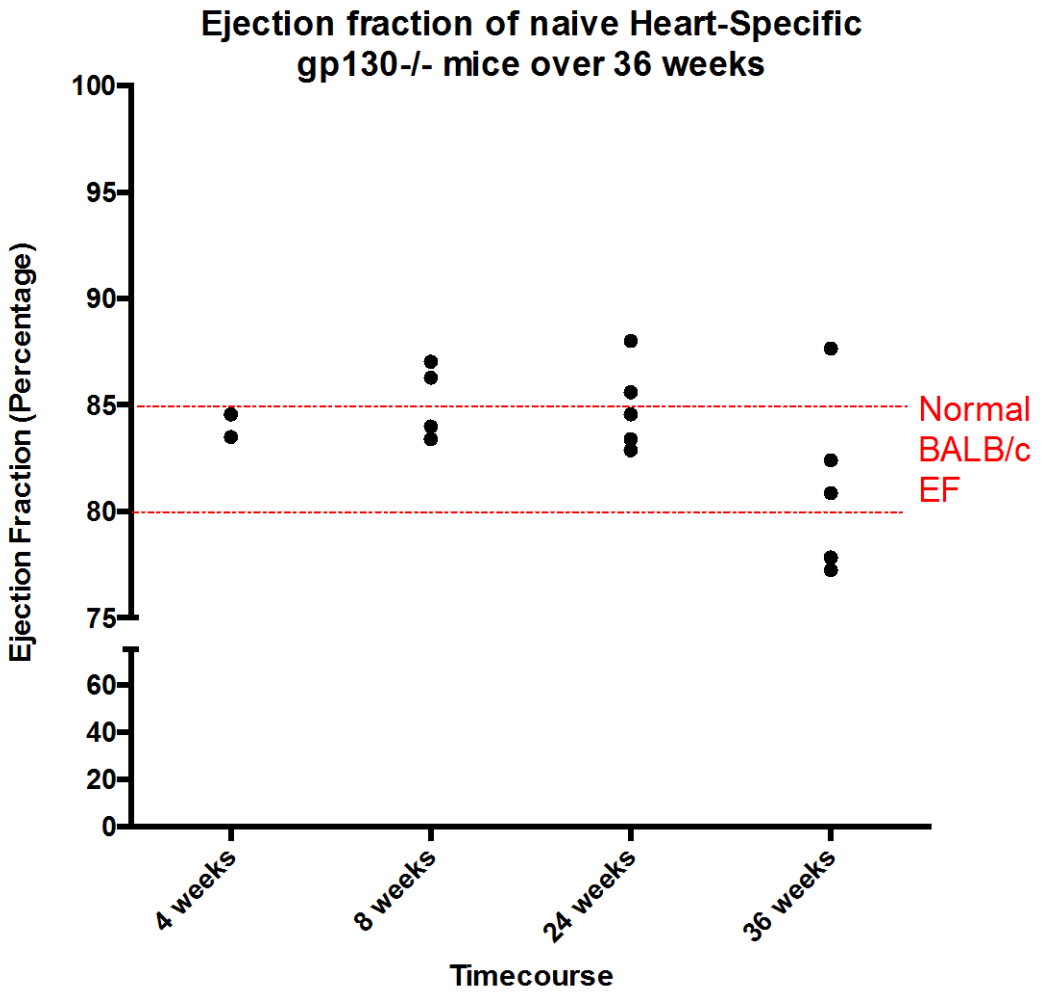

Figure 3.7 GP CKO mice have normal heart function over time

Naïve GP CKO mice were assessed for heart function, serially, over 36 weeks by echocardiography for ejection fraction (EF).

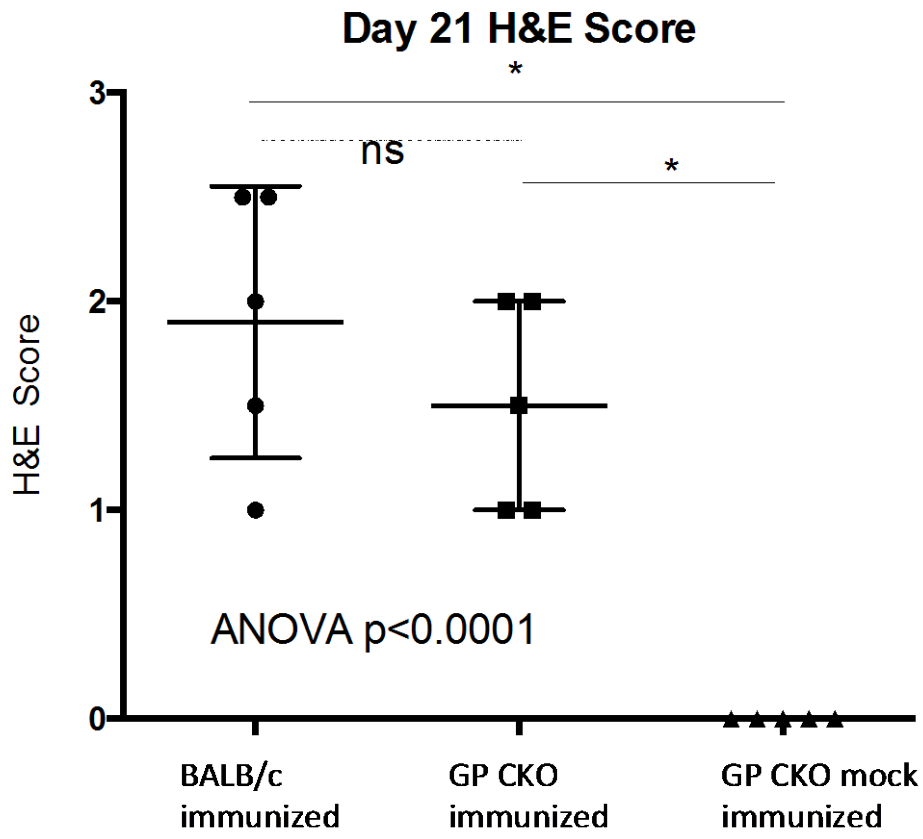

Figure 3.8 EAM develops in GP CKO mice

EAM was induced in WT BALB/c mice, GP CKO mice or mock-immunized GP CKO mice. Mice were sacrificed 21 days post-immunization. EAM was assessed by histopathology. Histopathological scoring of EAM in mice hearts scored using H&E staining. Data points represent individual mice. Bars represent mean. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$. Data are representative of 3 independent experiments.

Figure 3.9 BALB/c and GP CKO mice develop similar heart-infiltrating populations during EAM

EAM was induced in WT BALB/c mice or GP CKO mice. Mice were sacrificed 21 days post-immunization. EAM was assessed by heart-infiltrating cells and T-cell subsets and analyzed by flow cytometry.

(A) Total intracardiac CD45+ leukocytes in mice hearts. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. Data are representative of 3 independent experiments.

(B) Total intracardiac CD4+ cells in mice hearts expressed as a percentage of total CD45+ cells. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. Data are representative of 3 independent experiments.

(C-E) Intracellular cytokine staining of total intracardiac CD4+ cells in mice hearts. Data points represent individual mice. Bars represent mean. Data are analyzed by student's T-test. Data are representative of 3 independent experiments.

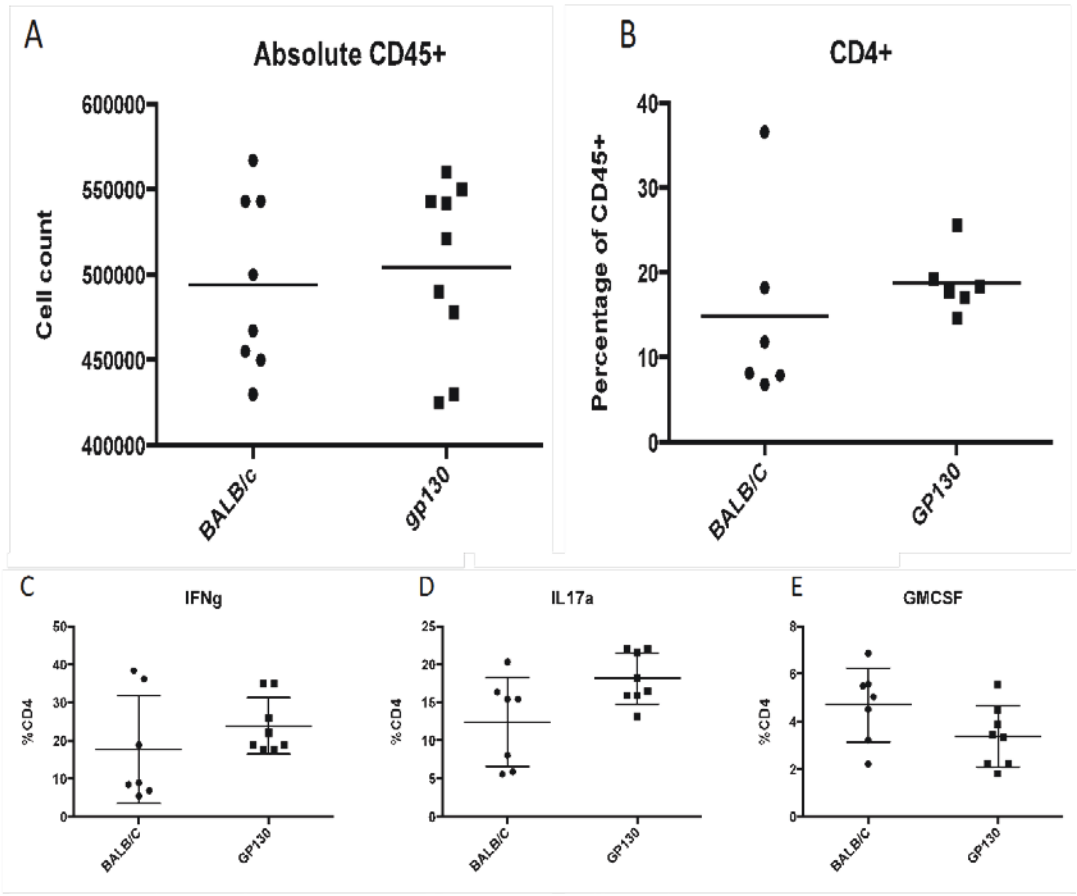

Figure 3.9 BALB/c and GP CKO mice develop similar heart-infiltrating populations during EAM

Figure 3.10. IL-6 signaling to the cardiomyocyte is required for progression to DCM

EAM was induced in WT BALB/c mice, GP CKO mice or mock-immunized GP CKO mice. Mice were sacrificed 45 days post-immunization. DCM was assessed by histopathology and echocardiography.

(A) Histopathological scoring of DCM in mice hearts scored using Masson's trichrome blue staining. Data points represent individual mice. Bars represent mean. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$. Data are representative of 3 independent experiments.

(B-C) Echocardiography to assess heart function of ejection fraction (EF) and left ventricle end systolic diameter (LVESd). Data points represent individual mice. Bars represent mean. Data are analyzed by one-way ANOVA followed by Tukey's post-test. *, $p<0.05$. Data are representative of 3 independent experiments.

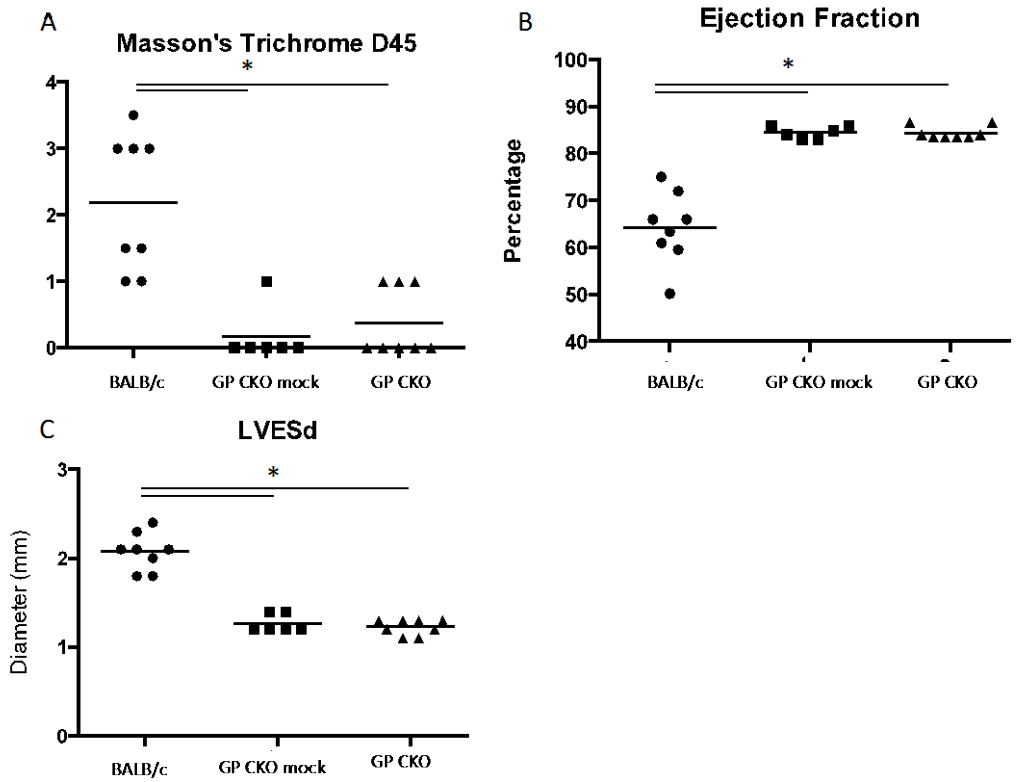

Figure 3.10. IL-6 signaling to the cardiomyocyte is required for progression to DCM

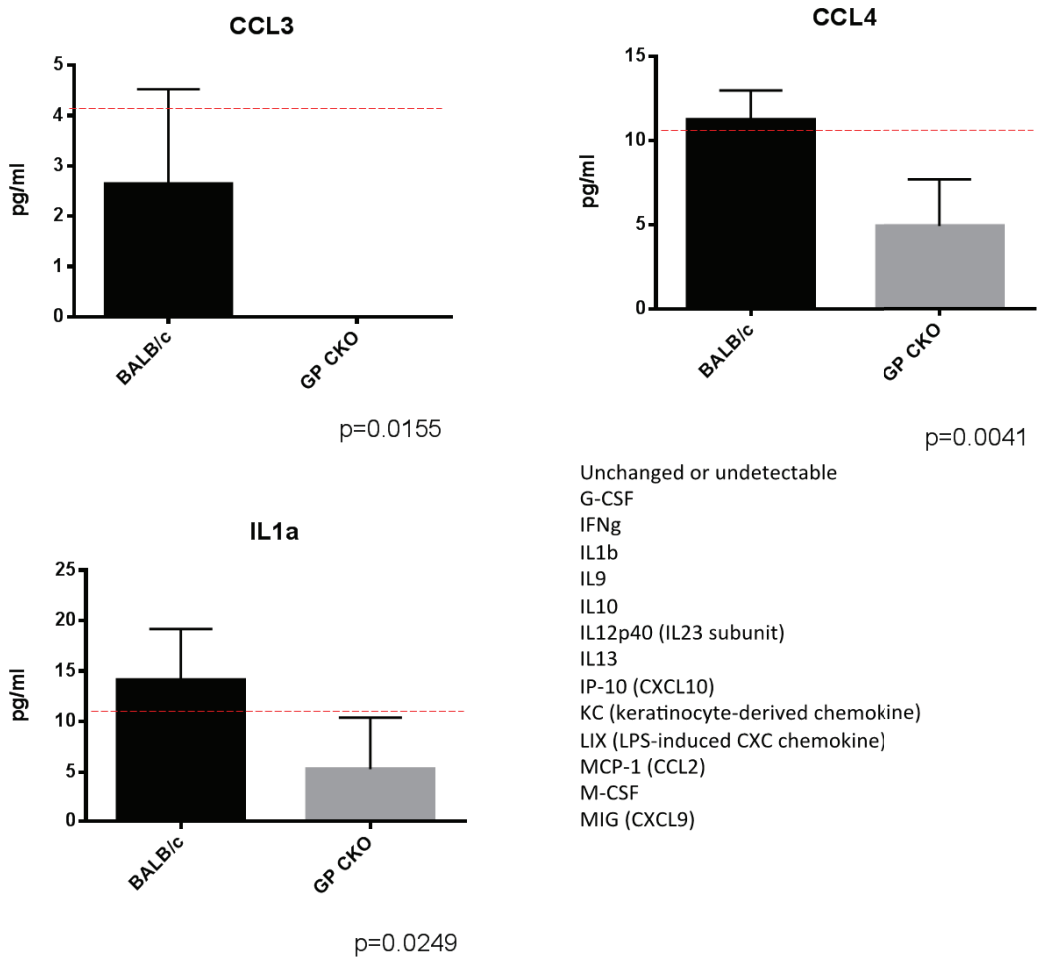

Figure 3.11 GP-CKO Myocytes have decreased production of CCL3, CCL4 and IL-1a in response to IL-6 *in vitro*.

Primary adult mouse cardiomyocytes from BALB/c or GP CKO naïve adult mice were cultured with rIL6+sIL6R for 24 hours. Supernatant was collected after culture, and the levels of chemokines and cytokines were measured by multiplex bead array. The red dotted lines represent the threshold level of detection of the assay.

Figure 3.12. Myocytes require IL-6 signaling in trans rather than classical signaling in vitro

Primary adult mouse cardiomyocytes from BALB/c or GP CKO naïve adult mice were cultured for 24 hours. Supernatant were collected after culture, and the levels of chemokines and cytokines were measured by multiplex bead array. The red dotted lines represent the threshold level of detection of the assay.

(A) In order to test the viability and responsiveness of the myocytes, myocytes were treated with IL-1b as a positive control.

(B) In order to test whether the myocytes signaled through classical IL-6 signaling (IL-6 alone) or trans signaling (sIL-6R+IL-6) myocytes were treated with IL-6 alone or sIL6-R+IL6.

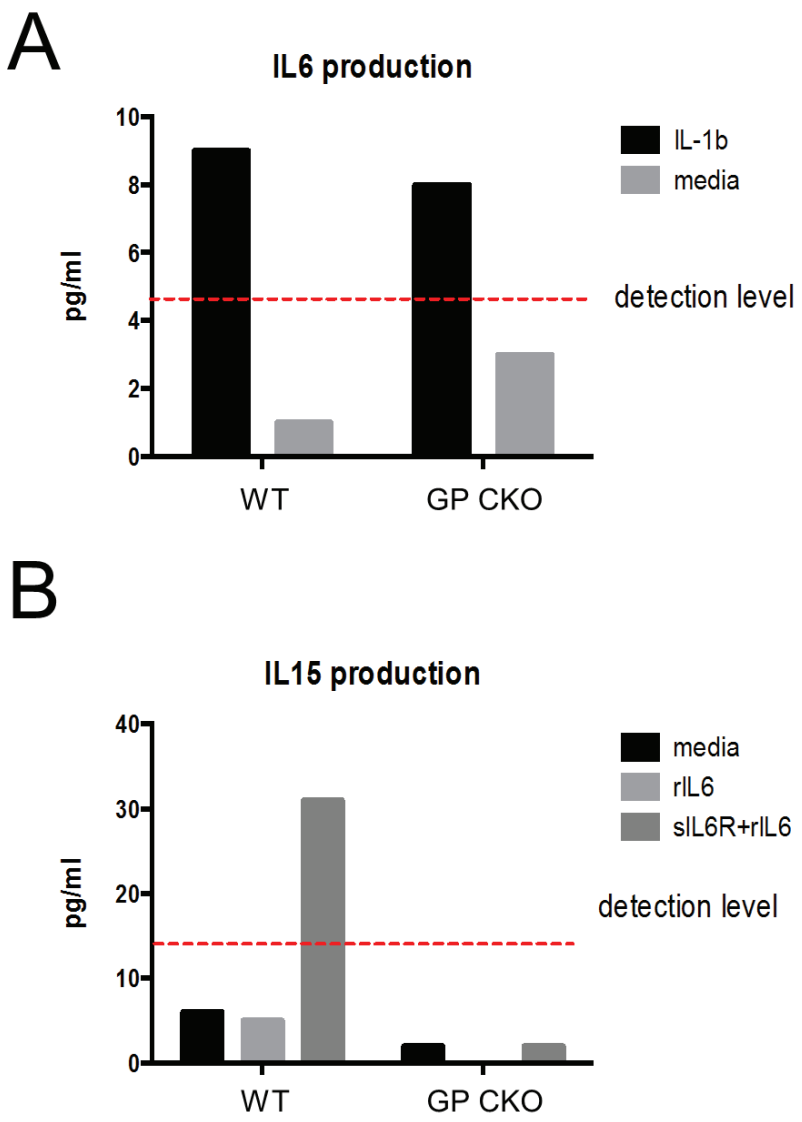

Figure 3.12 Myocytes require IL-6 signaling in trans rather than classical signaling in vitro

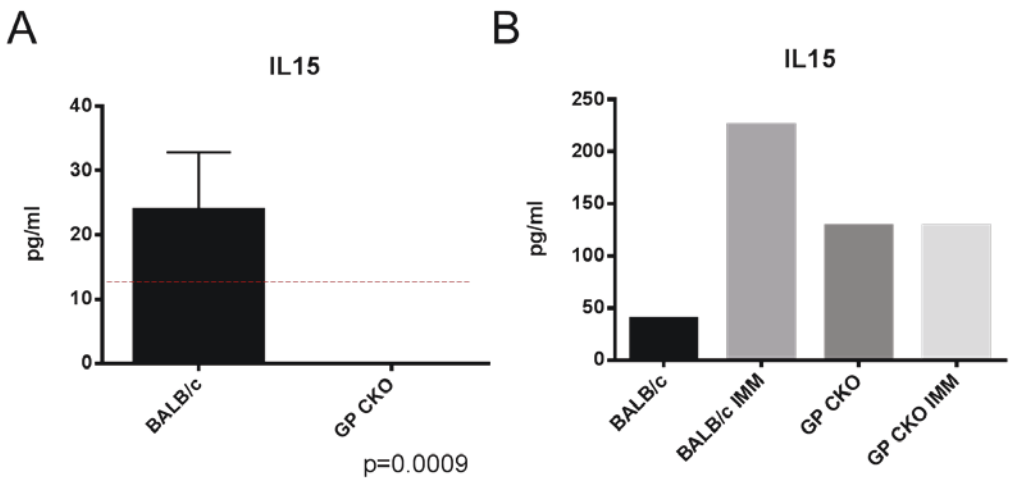

Figure 3.13 IL-6 induces IL-15 production by the cardiomyocyte

IL-15 induction in vitro and vivo by cardiac myocytes.

(A) Primary adult mouse cardiomyocytes from BALB/c or GP CKO naïve adult mice were cultured with rIL6+sIL6R for 24 hours. Supernatant were collected after culture, and the levels of IL-15 were measured by multiplex bead array. The red dotted lines represent the threshold level of detection of the assay.

(B) Mouse heart homogenates from naïve or EAM induced mice at day 21. EAM was induced in WT BALB/c mice or GP CKO mice. Mice were sacrificed 21 days post-immunization or age-matched for naïve mice. IL-15 levels were assessed from whole heart homogenate by multiplex bead array.

Figure 3.14 IL-6 signaling on the cardiomyocyte alters the profile of heart-function-related proteins

EAM was induced in WT BALB/c mice or GP CKO mice. Mice were sacrificed 45 days post-immunization. Whole heart homogenate was prepared for Western blot analysis. These blots are representative of 3 independent experiments.

(A) Western blot immunostained for sarcoendoplasmic reticulum calcium transport ATPase (SERCA-2). BALB/c mice have less SERCA-2 than GP CKO mice. Secondary band in addition to 110 kDa band is a known background band (abcam).

(B) Western blot immunostained for brain natriuretic peptide BNP. BALB/c mice have more BNP than GP CKO mice.

(C) Western blot immunostained for atrial natriuretic peptide ANP. BALB/c mice have more ANP than GP CKO mice.

Isolated d21EAM WT BALB/c myocytes are a positive control for ANP staining.

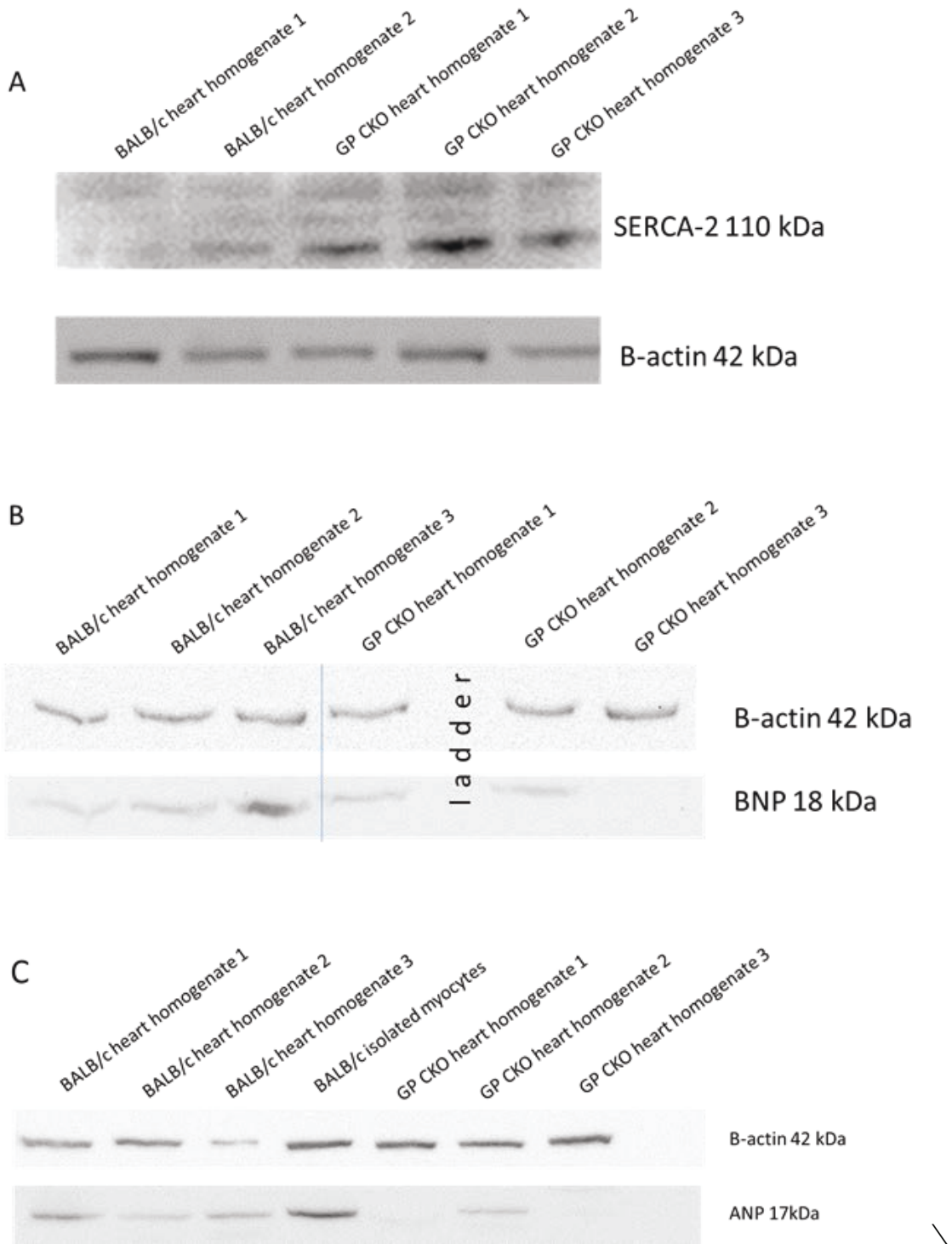

Figure 3.14 IL-6 signaling on the cardiomyocyte alters the profile of heart-function-related proteins

Chapter 4: Discussion

4.1 General Overview

Myocarditis and the consequences of this inflammation on heart function is a leading cause of sudden death in young adults [18]. Unresolved myocarditis can progress to DCM, a chronic disease that may result in heart failure. Myocarditis and myocarditis-associated DCM account for 45% of all heart transplants required in the United States [28]. The mechanisms driving the progression from myocarditis to heart failure are largely unknown. Identifying biomarkers of disease progression or targets of therapeutic intervention are needed for patient management. Currently, DCM is only cured by heart transplantation and management is restricted to general heart failure drugs for symptom treatment [27].

Increased IL-6 levels are associated with heart failure and are found in patients with DCM [105]. IL-6 levels represent an independent risk factor for CVD [91]. Employing the mouse model of myocarditis, EAM, the results presented in this dissertation has demonstrated the mechanisms by which IL-6 induces myocarditis and drives its progression to dilated cardiomyopathy. Chronically elevated IL-6 levels are a surrogate marker of disease and suggest a potential therapeutic target in disease, sIL-6R.

4.2 IL-6 in the priming phase of EAM

In addition to the identification of the role IL-6 plays in the progression of myocarditis to heart failure, this dissertation has described the critical role of IL-6 in the induction of myocarditis. In chapter 2 some of the basic steps by which IL-6 acts to promote the immune response to cardiac myosin-peptide are explored and shows for the first time that IL-6 enhances and broadens the response to adjuvant.

IL-6KO mice are known to be resistant to EAM induction [128]. Using anti-IL-6R antibodies we proved that IL-6 is required to induce EAM in mice that have developed in an IL-6 sufficient environment. Previous research has shown that IL-6KO mice do not develop any immune cell infiltration following immunization, but have not explained why or when IL-6 is required.

Using IL-6KO mice treated with recombinant IL-6, we isolated the role of IL-6 in the initial priming phase of EAM. IL-6KO mice treated with recombinant IL-6 cytokine for only 4 days surrounding the first immunization developed robust EAM not previously reported in IL-6KO mice. This determined the window in which IL-6 is needed for the pathogenic development of an autoimmune reaction to the heart. We conclude that IL-6 shapes the initial immune response to cardiac myosin peptide that will later develop into an immune reaction against the myocardium.

4.2.1 IL-6 and the response to adjuvant

Since IL-6 is required for the response to immunization, we sought to determine if IL-6 is a required factor for a response to adjuvant. It is known that the adjuvants commonly used for mice immunizations, CFA and IFA, induce very different responses during EAM [171]. CFA immunization is used to induce EAM because IFA immunization does not induce any disease in the heart. IFA immunization will induce antibody responses to immunization, as myosin-reactive antibodies can be measured following IFA immunization, but no EAM develops. EAM is T-cell driven and antibodies alone cannot induce disease [12, 13].

Supplementing IFA with rIL-6 at either first immunization on day 0 or at the boost on day 7, we show that the EAM development, and a robust and pathogenic immune response to the heart, is partially dependent upon IL-6. Mice immunized with IFA that received a single injection of recombinant IL-6 at the time of immunization developed immune-cell infiltration into the heart. The level of this EAM development was not equivalent to CFA-induced EAM, implying that other factors in addition to IL-6 are induced by CFA and lead to severe EAM.

4.2.2 IL-6 drives the local immune response through dendritic cells

We established that IL-6 was required, in low doses, for the priming phase of EAM induction. We then sought to determine the mechanism why IL-6 was

required for EAM. Using WT-BALB/c mice treated with anti-IL-6R antibodies or isotype control antibodies, we examined the local lymph node response to IL-6 signaling during immunization. We pre-treated mice with antibodies to create an environment either sufficient or void of IL-6R signaling and then immunized the mice with peptide-CFA. Three days following immunization we interrogated the chemokines and cytokines produced, and the cellular response in the draining lymph node. Through these experiments, we demonstrated that IL-6 is required to induce dendritic cell (DC) trafficking to the lymph node shown by DC CCR7 expression and lymph node CCL21 expression. This finding supports previous reports that IL-6 induced CCL21 expression by lymph node endothelial cells is required for CCR7+ DC trafficking [194].

In Chapter 2 we report that IL-6 is required for the initial response to immunization in order to induce DC responses in the local lymph node. We further show that IL-6 is required for an immune response to adjuvant that induces T-cell, rather than solely B-cell, responses, leading to EAM. These experiments clarify the role of IL-6, not only in EAM, but also as applied to adjuvant uses, such as vaccine development. Identifying that the induction of IL-6 in response to adjuvant changes a limited, B-cell response, into a pathogenic, T-cell involved response, can be incredibly informative for designing ideal immune responses to vaccines. Alternatively, this knowledge could be used to try and intervene in a pathogenic response to self, by

targeting IL-6 induced pathogenic responses dependent on DC sub-populations.

4.3 IL-6 in progression of EAM to DCM

After describing the role of IL-6 in disease induction we examined the role of IL-6 in the progression of myocarditis to DCM.

Using anti-IL-6R antibodies in WT BALB/c mice, we discovered that IL-6 is required for the development of DCM, as mice receiving the anti-IL-6R antibodies were protected from DCM even after EAM had been established. Treatment by anti-IL-6R antibodies during effector stage of EAM was an important experiment in order to show the therapeutic potential of targeting IL-6 without having to intervene in the initial immune response to cardiac myosin.

In order to confirm that IL-6 is critical in driving DCM, we approached the question by an opposite experiment. If blocking IL-6 protected from DCM development, could increasing IL-6 production worsen DCM?

Using hydrodynamic gene delivery of plasmid-IL-6 to mice with EAM, we showed that IL-6 drives functional parameters of heart failure while sparing further cardiac fibrosis. Mice that expressed higher than WT levels of IL-6 after EAM was established had diminished cardiac function, as characterized by a decreased ejection fraction, fractional shortening and more dilated hearts as measured by left ventricle end diastolic diameter and left ventricle

end systolic diameter. Thus, elevated IL-6 levels systemically contributed to heart dilation and failure. Both groups, regardless of plasmid insert, developed expected levels of fibrosis in the heart. But, the group with the higher IL-6 levels did not develop worse fibrosis. This novel finding suggests that the heart failure was not driven by enhanced fibrosis, but rather could depend upon the cell responsible for heart pumping function, the myocyte.

4.3.1 IL-6 signaling in the heart drives DCM through its depressive effects on the cardiac myocyte

In order to study the IL-6 contribution to DCM on the myocyte alone, we utilized myocyte-restricted gp130$^{-/-}$, GP CKO mice. These mice are globally IL-6 sufficient, but lack IL-6 signaling to the myocyte alone, creating a unique tool to study myocyte functions in vivo.

Using these mice, in Chapter 3 we demonstrated that the detrimental action of IL-6 in DCM development requires IL-6-mediated signaling in the myocyte. GP CKO mice are completely protected from DCM development. These mice have been extensively characterized; their hearts are developmentally normal, and have normal heart function [158, 172, 173]. We showed that over 36 weeks time GP CKO mice have normal heart function as compared with WT BALB/c mice. These mice are also susceptible to EAM, as they develop disease comparable to WT. However, despite their

unremarkable naïve phenotype, GP CKO mice are completely resistant to DCM.

We then sought to determine how IL-6 acted directly on the myocyte. We hypothesized that IL-6 directly affected the contractile function of the cell because IL-6 is one of the few cytokines that is known to act on myocytes. We also investigated the alternative hypothesis that inflammatory cells infiltrating the GP CKO hearts were involved, indirectly leading to the DCM protection. We also considered the possibility that fibrotic markers changed during earlier times during EAM than we had previously studied. To test these alternatives, we investigated the profile of immune infiltrating populations at day 21 in WT BALB/c and GP CKO hearts, finding no differences in proportion of innate and adaptive immune cells, including comparable proportion of Th1 and Th17 cells. We then interrogated markers of fibrosis by functional protein and mRNA levels at both day 14 and 21 of EAM. Again, there were no differences in fibrosis, lending further evidence to the role of IL-6 directly on the myocyte. After accepting the null hypothesis for both alternatives, we investigated changes to the myocyte itself.

By studying myocytes from GP CKO mice, we demonstrated that IL-6 signaling on the myocyte during DCM induces changes in the expression of proteins related to myocyte function. We examined 3 proteins known to relate to myocyte function, SERCA2, ANP and BNP. SERCA2 is a

sarcoendoplasmic reticulum calcium transport ATPase-2. SERCA2 is the heart isoform within SERCA proteins. We focused on this protein because it is required for myocyte excitation and contraction and its expression is known to be decreased in patients with heart failure [176]. Additionally, SERCA gene transfer has been experimentally shown to restore contractile function in failing hearts [190]. SERCA2 is a direct protein indicator of how well the myocyte is functioning, as decreases in SERCA2 levels mean less contractility. ANP and BNP are released from myocytes in response to mechanical stress and levels increase in response to changes in heart filing pressures [181]. Additionally, the release of ANP and BNP is increased in patients with heart failure [183]. ANP and BNP are both protective for the heart; they are released in order to return the myocyte to normal function [183]. However, their release is also indicative of stress and damage to the myocyte and therefore an indirect measure of myocyte dysfunction. By measuring these 3 proteins, 1 direct indicator of function, SERCA2, and 2 indirect indicators, ANP and BNP, we show that IL-6 alters myocyte function. Hearts from GP CKO mice, protected from DCM, had more SERCA2 and less ANP and BNP levels in the heart as analyzed by western blot of heart homogenate. Thus in WT-BALB/c hearts, IL-6 decreases SERCA2 expression in the myocyte and contractility, while increasing the expression of ANP and BNP, increasing myocyte stress. This result

establishes that the pathogenesis of IL-6 in DCM can be attributed in part to signaling on the myocyte leading to loss of cardiac function.

4.3.2 A model of IL-6 in EAM and DCM

In summary, we show a dual role of IL-6 in myocarditis. First it induces an appropriate innate response during the priming phase of EAM, then drives the progression of established EAM to DCM through IL-6 signaling-mediated loss of myocyte function.

Initially, IL-6 is produced in response to an inflammation-inducing insult, in our case CFA, and induces local responses which in turn leads to changes in the activation of local dendritic cells and the induction of cells in the draining lymph nodes to produce of CCL21. The induction of IL-6 is critical for eventual heart involvement in EAM as adjuvants that do not induce sufficient IL-6, such as IFA, do not induce EAM. The requirement for IL-6 in disease induction is limited to these initial reactions and is not needed further for EAM.

Once EAM is established, the second role of IL-6 is driving the disease from cardiac inflammation to heart failure. Chronically IL-6 acts upon the myocyte resulting in the loss of heart function and ultimately DCM. As IL-6 is required to signal in trans to myocytes because they do not express the membrane-bound IL-6R, these experiments present a possible therapeutic

target for preserving heart function in patients at risk of myocarditis-associated DCM.

4.4 IL-6 signaling in the heart: From acute and protective to chronic and pathogenic

Classically IL-6 is considered to be a proinflammatory cytokine. When homeostasis is disturbed within a host, IL-6 is elevated and induces protective responses determined by the nature of the insult. IL-6 can activate immune cells, direct immune cell trafficking, signal protective responses in local tissue, initiate the acute phase response or contribute to wound healing. In Chapter 2 we show how IL-6 is vital in directing the local response to immunization in the draining lymph node, which is ultimately required for eventual T-cell mediated responses in the heart.

These differing actions of IL-6 are all consequences of its basic biology (see Chapter 1). During an acute response IL-6 can enhance protective responses by increasing immune infiltration and enhancing wound repair. In the longer term, these actions of IL-6 can lead to inflammatory and fibrotic disorders. The heart is a tissue where this duality is dramatically illustrated. Studies from MI show how short-term IL-6 signaling can protect and preserve the heart tissue in response to acute damage, whereas long term IL-6 signaling or over-production plays a causal role in cardiovascular disease. In Chapter 3 we demonstrate how chronically elevated IL-6 levels induce heart failure

post-EAM and how knowledge of this process could be a target for intervention.

4.5 Translational opportunities

The identification of the unique nature of IL-6 signaling, which occurs through both classical, membrane-bound signaling and through signaling in trans, with a soluble form of the IL-6R, has created the opportunity for therapeutic intervention. Blocking all IL-6 signaling has severe consequences as IL-6 serves many vital functions. Anti-IL6 treatment is currently reserved only for severe cases of RA [116]. Having a method to only block the specifically pathogenic signals of IL-6 is an exciting avenue of future research. The current use of the available IL-6R antibody, which targets both classical and trans signaling, is limited, as tocilizumab is given monthly by intravenous (IV) infusion. However, many current animal studies aim to design more specific inhibitors, through the use of a soluble gp130 decoy, sgp130Fc, which inhibits only trans-signaling [195]. The research presented in this dissertation shows how patients with myocarditis may benefit from therapeutic agents such as sgp130Fc, which can prevent loss of myocyte function from chronic IL-6 signaling without globally suppressing immune responses.

4.6 Outstanding Questions and Future Research

Chapter 2 implicates dendritic cell changes in the response to adjuvant. Additionally, lymph node tissue was shown to upregulate DC trafficking markers. What remains to be answered is whether IL-6 is required for the appropriate migration alone or if IL-6 signaling to DCs affects DC presentation to T cells. Knowing how IL-6 alters DC function could be a way to fine-tune innate cell responses during immunization.

Chapter 3 elucidates how IL-6 signaling to myocytes drives progression to heart failure. Still to be answered is the cellular source of that IL-6 in the heart and whether systemic IL-6 levels, which correlate to disease progression, reflect the local production of IL-6. The results presented in Chapter 3 strongly suggest soluble-IL-6 signaling is a potential therapeutic target, therefore understanding the source and global IL-6 levels could inform treatment options. Global IL-6 signaling suppression blocks the protective and pathogenic outcomes of IL-6, therefore knowing how to target the local heart-specific IL-6 signaling could minimize the risks associated with blocking local IL-6 production or signaling.

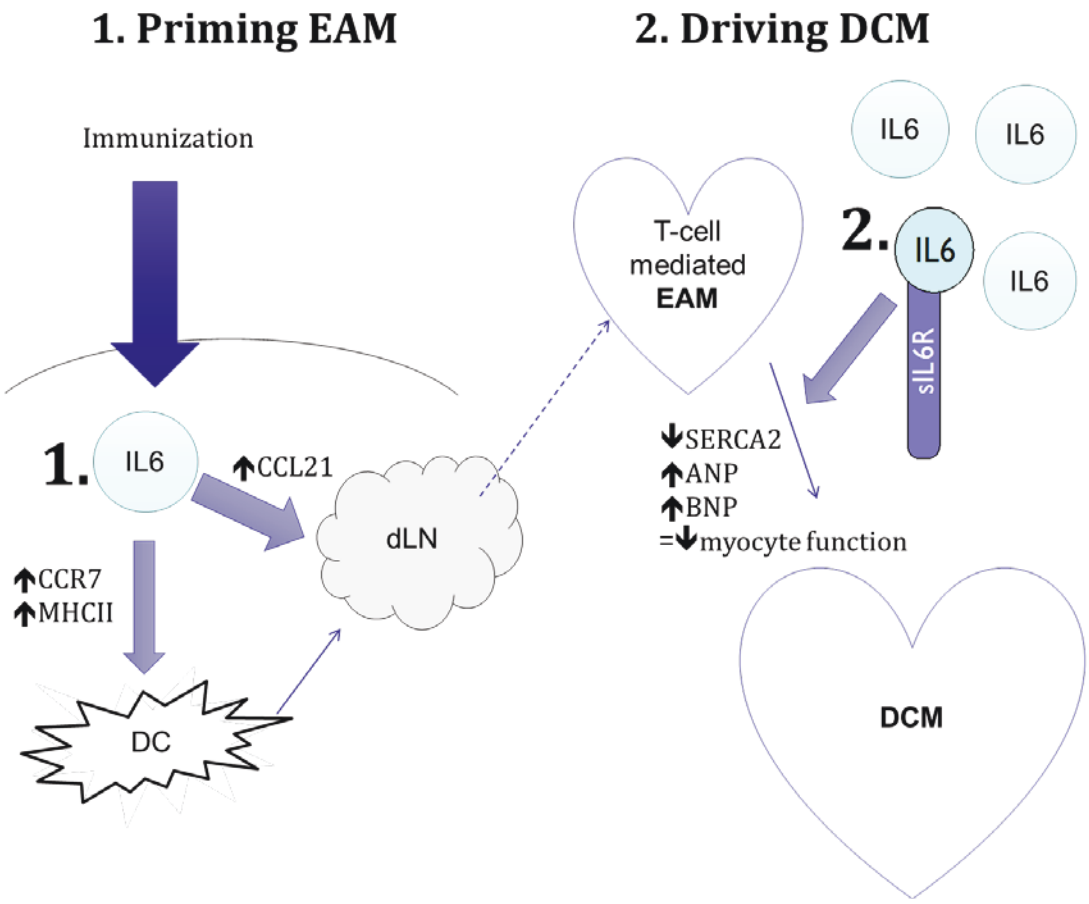

Figure 4.1. The dual role of IL-6 in priming EAM and in driving DCM

1. IL-6 is initially required for the increase of DC MHCII expression and DC CCR7 expression, as well as the increased of CCL21 expression by the draining lymph node in order for the eventual T-cell involvement driving EAM.

2. IL-6 is required for the progression of EAM to DCM by it's action on the myocyte. IL-6 decreases SERCA2 expression, increases ANP and BNP expression and depresses myocyte function leading to DCM.

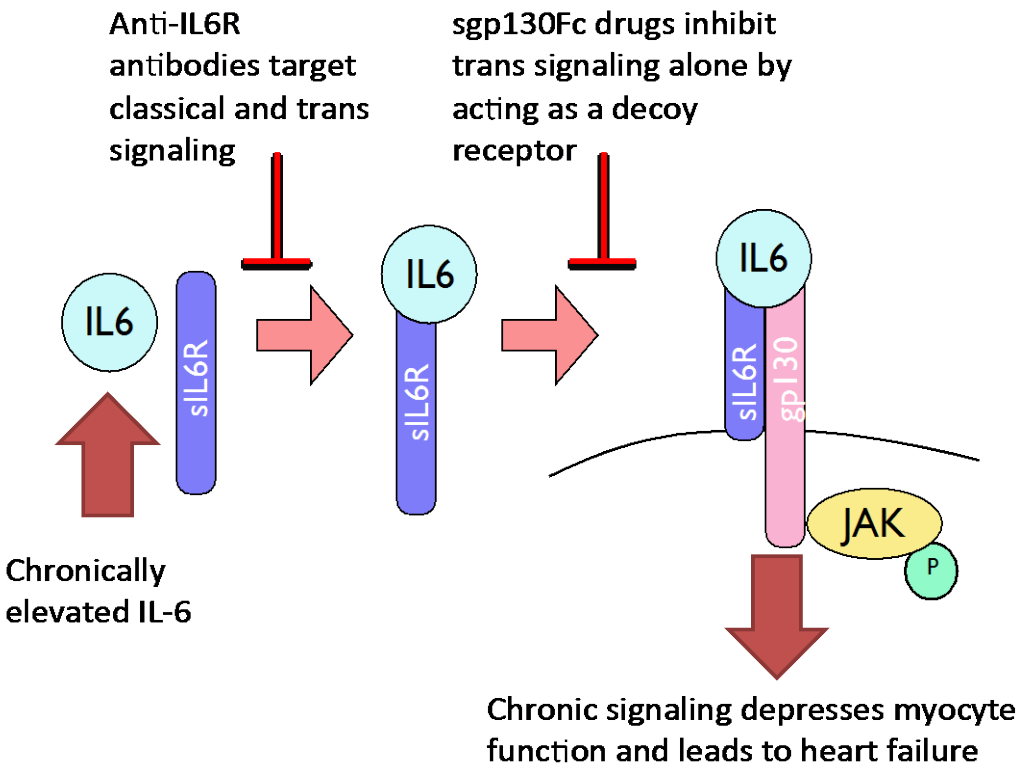

Figure 4.2. Therapeutic targets of chronic IL-6 signaling

IL-6 signaling can be blocked using antibodies. Antibodies to the IL-6R block both classical and trans signaling of IL-6 because the antibody will bind to the soluble or membrane-associated form of the receptor and block subsequent signaling. Alternatively, trans signaling can be blocked in isolation using a decoy gp130 receptor, sgp130Fc. This targets trans signaling alone because the soluble IL-6R bound with IL-6 is the complex which binds with sgp130Fc, thus isolating sIL-6R mediated, trans, signaling. Using either anti-IL6R antibodies of sgp130Fc blockade, the pathogenic signaling of IL-6 is inhibited, however, sgp130Fc spares the classical IL-6 signaling, which is not associated with heart failure.

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Curriculum Vitae

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Date and Place of Birth

July 10th, 1985 in St.Catharines, Ontario, Canada

Education

Ph.D

Johns Hopkins Bloomberg School of Public Health, Baltimore MD

Harry W Feinstone Department of Molecular Microbiology and Immunology

Thesis: ***The dual role of IL-6 in autoimmune myocarditis and inflammatory dilated cardiomyopathy***

Mentor: Noel R Rose, MD, PhD

M.S.

University of California, Davis, Davis CA

USDA, Western Human Nutrition Research Center

Nutritional Biology, September 2009

Thesis: ***The 5-lipoxygenase pathway in human monocytes before and after an omega-3 fatty acid intervention trial***

Mentor: Charles Stephensen, PhD

B.A.

Cornell University, **Ithaca NY**

College of Arts and Sciences

Biology and Society, May 2007

<u>Awards and Honors</u>

Gordis Teaching Fellow 2014 and 2015

Johns Hopkins Krieger School of Arts & Sciences

and Johns Hopkins Bloomberg School of Public Health

Fellowship to design and lead my own course for senior undergraduate students, entitled:

'Nutrition and Immunology in Chronic Disease'. Taught in both Fall and Spring semesters.

Excellence in Basic Research, 16th Annual Pathology Young Investigator's Day Award 2014

Department of Pathology

Johns Hopkins University School of Medicine

"The dual role of IL-6 in priming autoimmune myocarditis and driving inflammatory dilated cardiomyopathy"

Excellence in Basic Research, 15th Annual Pathology Young Investigator's Day Award 2013

Department of Pathology

Johns Hopkins University School of Medicine

"IL-6 promotes the progression of experimental autoimmune myocarditis to dilated cardiomyopathy"

Martin Frobisher Fellowship Award 2010

Department of Molecular Microbiology and Immunology,

Johns Hopkins Bloomberg School of Public Health

Publications (Note: Name change, previously JA Legault, now JA Fontes)

Jillian A Fontes, Noel R Rose and Daniela Cihakova. The varying faces of IL-6: from cardiac protection to cardiac failure. Cytokines. *Accepted, in publication.*

SuFey Ong, Davinna L Ligons, Jobert G Barin, Lei Wu, Monica V Talor, Nicola Diny, **Jillian A Fontes,** Elizabeth Gebremariam, David A Kass, Noel R Rose, Daniela Čiháková. Natural killer cells limit cardiac inflammation and fibrosis by halting eosinophil infiltration. American Journal of Pathology. *Accepted, in publication.*

Barin JG, Baldeviano GC, Talor MV, Wu L, Ong S, Fairweather D, Bedja D, Stickel NR, **Fontes JA**, Cardamone AB, Zheng D, Gabrielson KL, Rose NR, Ciháková D. Fatal eosinophilic myocarditis develops in the absence of IFN-γ and IL-17A. J Immunol. 2013 Oct 15;191(8):4038-47. doi: 10.4049/jimmunol.1301282. Epub 2013 Sep 18.

Jillian A. Legault, G. Christian Baldeviano, SuFey Ong, Lei Wu, Jobert G. Barin, Monica V. Talor, Daniela Cihakova, Noel R. Rose. ABSTRACT 161 : IL-6 promotes the progression of experimental autoimmune myocarditis to dilated cardiomyopathy, Cytokine, Volume 63, Issue 3, September 2013, Page 281, ISSN 1043-4666, http://dx.doi.org/10.1016/j.cyto.2013.06.164.

Barin JG, Baldeviano GC, Talor MV, Wu L, Ong S, **Legault JA**, Zheng D, Caturegli P, Rose NR, Cihákova D. Fatal eosinophilic myocarditis develops in the absence of IFNγ and IL17A. J Immunol. 2013 Oct 15;191(8):4038-47. doi: 10.4049/jimmunol.1301282. Epub 2013 Sep 18.

Jillian Legault, G. Baldeviano, Jobert Barin, Lei Wu, SuFey Ong, Monica Talor, Daniela Cihakova,and Noel Rose. IL-6 is necessary for the progression of experimental autoimmune myocarditis to dilated cardiomyopathy. *J Immunol 2012 188:171.11.* ABSTRACT, AAI Meeting.

Charles B. Stephensen, Armstrong P, Newman JW, Pedersen T, **Legault J**, Schuster G, Kelley D,Vikman S, Hartiala J and Hooman Allayee. ALOX5 gene variants affect eicosanoid production and response to fish oil supplementation. Journal of Lipid Research, 2011 May;52(5):991-1003.Epub 2011 Feb 4.

Presentations

Meetings and Conferences

Heart & Vascular Institute Annual Cardiovascular Research Retreat,

Baltimore MD May 30, 2014

Johns Hopkins Heart and Vascular Institute

Poster Presentation: The dual role of IL-6 in priming autoimmune myocarditis and driving inflammatory dilated cardiomyopathy

Department of Pathology Young Investigators' Day,

Baltimore, MD April 3, 2014

Johns Hopkins Medical Institutions

Poster Presentation: The dual role of IL-6 in priming autoimmune myocarditis and driving inflammatory dilated cardiomyopathy

Cytokines 2013, San Francisco, CA September 28, 2013

International Cytokine Society and International Society for Interferon and Cytokine Research

Poster Presentation: IL-6 drives the progression of experimental autoimmune myocarditis to dilated cardiomyopathy

Heart & Vascular Institute Annual Cardiovascular Research Retreat,

Baltimore MD May 31, 2013

Johns Hopkins Heart and Vascular Institute

Poster Presentation: IL-6 promotes the progression of experimental autoimmune myocarditis to dilated cardiomyopathy

Department of Pathology Young Investigators' Day,

Baltimore, MD April 5, 2013

Johns Hopkins Medical Institutions

Poster Presentation: IL-6 promotes the progression of experimental autoimmune myocarditis to dilated cardiomyopathy

American Association of Immunology Annual Meeting,

Boston, MA May 6, 2012

Poster Presentation: IL-6 is necessary for the progression of experimental autoimmune myocarditis to dilated cardiomyopathy

Teaching Experience

Johns Hopkins Krieger School of Arts & Sciences

Instructor, 2014-2015

Course title: Nutrition and Immunology in Chronic Disease

Gordis Teaching Fellowship to design and teach a course for undergraduate junior and senior students in the Public Health Major. Bi-weekly, semester

long, seminar course aimed at teaching scientific critical thinking and critical analysis of scientific results through the topic of the interaction of diet and the immune system in chronic disease. Taught Fall 2014 and Spring 2015.

Johns Hopkins Bloomberg School of Public Health

Lead Teaching Assistant, 2010-2014

Introduction to the biomedical sciences

Team-taught, 2-week intensive course for entering students at the School of Public Health

Teaching Assistant, 2010-2015

Infection, Immunity and Undernutrition: Interactions and Effects *(distance education), 2012-2015*

Public Health Biology *(distance education), 2013, 2014*

Fundamental Virology *2010*

University of California, Davis: Department of Neurobiology, Physiology and Behavior

Teaching Assistant and Laboratory Instructor, 2008 – 2009

Advanced systemic physiology laboratory *(Upper division)*

Cellular physiology/neurobiology laboratory *(Upper division)*

Systemic physiology laboratory *(Upper division)*

<u>Professional Training</u>

Teaching at the University Level Course

Johns Hopkins Bloomberg School of Public Health, 2014

Fundamentals of Fluorescence and Confocal Microscopy Course

Johns Hopkins Medical Institutions, 2011

Mouse Pathobiology and Phenotyping Summer Workshop: Mouse Biology, Pathology, Genetics for Phenotyping and Translational Research

Johns Hopkins Medical Institutions, 2011

Bioinformatics and Genomics Course; Database Utilization

Johns Hopkins Medical Institutions, 2010